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ISOIMMUNITY TO THE RH FACTOR AS A CAUSE OF BLOOD TRANSFUSION REACTIONS

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IN 1939 Levine and Stetson[1] reported the case of a woman who, after delivering a macerated fetus, developed a hemolytic reaction to a transfusion of blood from her husband. The blood of the husband and wife belonged to group O, but the recipient's serum was shown to contain an agglutinin which acted against the cells of about 80 per cent of persons belonging to Group O. The agglutinable factor was not M, N, or P. The potency of the agglutinin was diminished in two months and had disappeared from the blood in one year. It was concluded that the antibody was an isoimmune phenomenon which might have resulted from disintegration products from the dead fetus. Injections of agglutinable blood cells into rabbits failed to yield an antiserum. Landsteiner and Wiener,[2] in 1940, using agglutinin sera prepared by injecting rabbits with erythrocytes of rhesus monkeys, demonstrated antibodies exhibiting specificity apparently similar to the serum described by Levine and Stetson. The rabbit serum agglutinated the cells from approximately 85 per cent of human beings. They designated the agglutinable factor by the initials Rh. In the same year, Wiener and Peters[3] reported three cases in which repeated transfusions of Rh-positive blood into Rh-negative recipients stimulated the formation of anti-Rh agglutinins resulting in hemolytic reactions. Levine and coworkers[4] demonstrated the probability that erythroblastosis fetalis is caused by the development of antibodies in the Rh-negative mother against the Rh-positive blood of the fetus, resulting in a hemolytic type of anemia in the infant. They also described transfusion reactions in the mother from the presence of anti-Rh antibodies developed by pregnancy.[5]

Some individuals whose blood is Rh negative would seem to possess the potentiality of developing anti-Rh agglutinins by either of two mechanisms: by receiving blood in repeated transfusions from Rh-positive donors or by bearing children whose blood is Rh positive. Since approximately 15 per cent of persons of the white race in the United States have blood which is Rh negative, it might be considered that the development of isoimmunity to the Rh factor would be a common phenomenon through either of the two mechanisms. Actually, although the combination of Rh-positive child and Rh-negative mother occurs in about 1 of 10 pregnancies, only 1 pregnancy in 400 results in hemolytic disease of the child. It has also been noted that not all Rh-negative recipients are immunized by repeated transfusions of Rh-positive blood.

From the Department of Internal Medicine, University Hospitals.
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It is particularly desirable to obtain some perspective on the incidence of isoimmunity in a general transfusion service because the prevention of transfusion reactions due to the anti-Rh agglutinin requires special laboratory tests preliminary to transfusion. The military employment of multiple transfusions of Group O donors to recipients of any blood group without preliminary cross-matching also raises the question of possible mortality from isoimmunization to the Rh factor.

METHODS OF INVESTIGATION

During a period of eighteen months (March 1, 1943, to Sept. 1, 1944) a series of 5,386 consecutive blood transfusions given to patients in the State University of Iowa Hospitals, under the supervision of staff members of the Blood Transfusion Service, was studied. The majority of the recipients were chronically ill. Transfusions were given without regard to the Rh type of the donor or the recipient. Many recipients received multiple transfusions. The obstetric history of female patients was disregarded, at least before transfusion. The recipients included both adults and children who were patients in a general hospital of approximately 800 beds. The blood of both donor and recipient was grouped with anti-A and anti-B sera of high titer, using the centrifuge technique. Most of the transfusions involved blood of homologous group. Preliminary cross-matching was performed at room temperature, the erythrocyte-serum suspensions being centrifuged and read within three minutes. In the great majority of cases, blood preserved in the modified Rous-Turner mixture[6] and stored from one to thirty days at from 4 to 6° C. was employed, although some transfusions of citrated blood, both fresh and stored, were given.

Most of the recipients were visited after transfusion by a member of the staff of the Blood Transfusion Service. Chills, fever, urticaria, dyspnea and other signs of reactions were noted. The post-transfusion urine was examined for hemoglobin. In cases of reaction associated with blood transfusion, the blood of the donor and recipient was typed for the Rh factor and the blood was cross-matched to determine the presence of anti-Rh agglutinins. These tests were performed by incubating a mixture of appropriate erythrocytes and serum for sixty minutes at 37° C., resuspending, and centrifuging for one minute. Then, after gentle shaking, the suspension was examined in the microscope for the presence of even small clumps.

CLASSIFICATION AND INCIDENCE OF REACTIONS

Reactions were classified according to the chief clinical manifestation. Many were characterized by chills and fever of only a few hours' duration. In these, the presence of hemoglobinemia was excluded by examination of the recipient's serum for hemoglobin and bilirubin. Urticarial reactions were easy to identify. Hemolytic reactions were diagnosed by the presence of hemoglobin in the post-transfusion urine or by the demonstration of free hemoglobin or increased bilirubin in the recipient's serum. Jaundice was determined by inspection. Gastrointestinal reactions were characterized by nausea and vomiting; their nature is uncertain. Cardiovascular reactions were evident by the clinical signs of left-sided cardiac embarrassment: dyspnea, cyanosis, coarse râles in the lungs, and prompt cessation after the application of tourniquets to the extremities.

The incidence of reactions due to all causes was as follows:

Transfusions without reactions	5,200	
Transfusions with reactions of all types	186	(3.4%)
Total transfusions	5,386	

The 186 transfusion reactions were classified as follows:

Chills and fever	101	(1.8%)
Urticaria	44	(0.8%)
Hemolysis	12	(0.2%)
Jaundice	8	(0.1%)
Gastrointestinal	11	(0.2%)
Cardiovascular	10	(0.1%)

In the 186 reactions of all types, but six were found which could be attributed to isoimmunity to the Rh agglutinogen. This constitutes an incidence of reactions due to the Rh factor of 0.1 per cent in the 5,386 blood transfusions. Isoimmunity was responsible for 3.2 per cent of the 186 reactions in the series. Sensitization to the Rh factor was attributed to multiple transfusions in four instances; pregnancies were responsible in the other two. There was one fatality.

TABLE I

BLOOD TRANSFUSION REACTIONS ANALYZED ACCORDING TO THE NUMBER PER RECIPIENT AND SEX OF THE RECIPIENT

NUMBER OF TRANSFUSIONS PER RECIPIENT	MALE RECIPIENTS			FEMALE RECIPIENTS		
	NUMBER OF RECIPIENTS	TOTAL TRANSFUSIONS	NUMBER OF REACTIONS	NUMBER OF RECIPIENTS	TOTAL TRANSFUSIONS	NUMBER OF REACTIONS
1	508	508	15	532	532	17
2	205	410	17	194	388	15*†
3	157	471	9	121	363	9‡
4	77	308	8§	61	244	15
5	53	265	6	32	160	9‖
6	32	192	4	19	114	7
7	24	168	4	13	91	1
8	10	80	1	10	80	6
9	10	90	3	1	9	1
10	7	70	3	5	50	1
11	4	44	0	5	55	0
12	3	36	2¶	4	48	4
13	4	52	1			
14	3	42	2	1	14	2
15	1	15	0			
16	3	48	10**	2	32	1
17	1	17	0	2	34	0
19	1	19	1	1	19	0
20	1	20	0			0
21	1	21	0	1	21	0
23	2	46	7			
24	1	24	1			
25	1	25	0			
28	1	28	0			
40	1	40	0			
93	1	93	4			
Total	1,112	3,132	98 ($3.1 \pm 0.2\%$)	1,004	2,254	88 ($3.9 \pm 0.4\%$)
	Total number of transfusions			5,386		
	Total number of reactions			186 ($3.4 \pm 0.2\%$)		

*Includes Case 44-1090, patient sensitized by pregnancies.
†Includes Case 44-6538, patient sensitized by transfusions not included in this series.
‡Includes Case 38-10586, patient probably sensitized by single pregnancy.
§Includes Case 43-11894, patient sensitized by transfusions.
‖Includes Case 38-42221, patient sensitized by transfusions.
¶Includes Case 38-15984, patient sensitized by transfusions.
**Includes seven urticarial reactions in one recipient.

In Table I are recorded the data on transfusion reactions distributed according to the sex of the recipients and the number of transfusions received by each. In any large group of recipients receiving multiple transfusions, the incidence of reactions due to isoimmunity to the Rh factor might be expected to be higher in females than in males because many of the former have had the added factor of pregnancy. There appears to be a statistically significant difference in the incidence of transfusion reactions of all types in the males and females of this series (3.1 ± 0.2 per cent as opposed to 3.9 ± 0.4 per cent), although two males and two females were actually shown to have been sensitized by previous transfusions. With improved serologic methods, a greater number of reactions might be proved to be due to isoimmunity to the Rh factor.

Analysis of the data sheds some light on the probability of the Rh-negative recipient becoming sensitized to the Rh factor by receiving multiple blood transfusions. No patient in this series developed isoimmunity with less than four transfusions. Taking four blood transfusions as a purely arbitrary minimum for purposes of calculation, there were 242 males and 157 females who received four or more transfusions. It can be assumed that approximately 15 per cent of the 399 recipients, or sixty persons, were Rh negative. Only four of these were proved to have become immunized to the Rh factor, an incidence of 6.6 per cent.

CASE HISTORIES OF PATIENTS SHOWING ISOIMMUNITY TO THE RH FACTOR

CASE 43-11894.—Man, physician, aged 37 years, Group O. A gastrectomy was performed for a duodenal ulcer. The patient received transfusions of homologous group blood Oct. 28 and Oct. 30, 1943, without reaction. Nov. 27, 1943, he received another transfusion which was accompanied by urticaria. A fourth transfusion Jan. 25, 1944, was associated with a chill and fever of 103° F. There were no further signs or symptoms. The blood of the last donor was found to be Rh positive. The recipient's blood was Rh negative, but no anti-Rh agglutinin could be demonstrated in the serum. Isoimmunity in this instance was attributed to repeated transfusions.

CASE 38-15984.—Man, aged 21 years, Group O. The patient was under treatment for tuberculosis of the left hip. In a period of a month, from Oct. 10, 1943, to Nov. 11, 1943, he received ten blood transfusions of homologous group. The first four transfusions were without incident. There were chills and fever with the fifth and sixth. No reaction followed the seventh and eighth. Violent chills and fever were associated with the ninth and tenth transfusions. The recipient's cells were Rh negative and the serum contained an anti-Rh agglutinin. The cells of the last two donors were Rh positive. The cells of the father of the patient were found to be Rh negative. Subsequently the son was given two transfusions of the father's blood without reaction.

CASE 38-42221.—Woman, aged 37 years, Group A. After an incomplete abortion, the patient received blood transfusions July 6, 7, and 11, 1944, without reactions. Aug. 1, 1944, another transfusion of homologous blood group was accompanied by violent chills, pain in the thighs, and temperature of 101.2° F. A small amount of free hemoglobin was present in the blood serum after transfusion and there was hemoglobinuria. The recipient's blood was demonstrated to be Rh negative and contained agglutinins against the Rh-positive blood of the last donor. Aug. 20, 1944, she received another transfusion of Rh-negative blood without reaction. The obstetric history was interesting. There had been nine pregnancies, all deliveries having been performed in the University Hospitals. The sixth pregnancy had resulted in a premature infant who died in the hospital without signs of erythroblastosis fetalis. The blood of seven living children was typed for the Rh factor. The children from the first, second, and fourth pregnancies were Rh positive; those from the third, fifth, seventh, and eighth

were all Rh negative. This was concluded to be another example of isoimmunity developing from repeated transfusions, although the occurrence of the abortion suggests a possible transplacental mechanism.

CASE 44-1090.—Woman, aged 49 years, Group B. During treatment for metrorrhagia from chronic cervicitis, the patient received a blood transfusion of homologous group Feb. 2, 1944. This was associated with chills and fever. The next day she was given a second transfusion which resulted in a violent chill, fever of 103.8° F., and transient jaundice which appeared in about six hours. The blood cells were found to be Rh negative and the serum contained anti-Rh agglutinins which strongly agglutinated the cells of many Rh-positive bloods by the centrifuge technique without incubation. The blood from the last donor was Rh positive. Three transfusions of Rh-negative blood were later given without reaction. The children from the first three pregnancies were living. In subsequent pregnancies, there had been three miscarriages, one stillbirth, and one child who died of umbilical hemorrhage two weeks after birth. It is probable that isoimmunity in this instance was due to repeated pregnancies, although the last had occurred ten years before.

CASE 38-10586.—Woman, aged 23 years, Group A. The patient was a primipara who entered the hospital with signs of toxemia of pregnancy. She had previously received a blood transfusion of homologous group Sept. 23, 1943, without reaction. Aug. 3, 1944, a second transfusion was given. When 350 c.c. of blood had been injected, the recipient had a chill, the temperature rose, and severe dyspnea developed. Coarse râles were present in the lungs. The transfusion was immediately discontinued and a specimen of the recipient's blood was promptly collected. The serum was yellow when contrasted with the normal color of the pretransfusion specimen. There was no free hemoglobin in the serum, but the van den Bergh reaction was 6.1 units, delayed direct. The urine for the next twenty-four hours contained no hemoglobin but was colored a deep brown with pigment which gave the chemical reactions for bilirubin. The transfusion reaction precipitated labor and the patient was delivered of a dead infant estimated to be eight months old. The child was somewhat jaundiced and the abdomen was distended with ascitic fluid. Blood taken from the right ventricle yielded a yellow serum with a van den Bergh reaction of 4.0 units, delayed direct. The baby's erythrocytes contained abnormally high numbers of normoblasts and megalocytes. The post-mortem findings were consistent with a diagnosis of erythroblastosis fetalis. The mother's blood cells were Rh negative and the infant's were Rh positive. The mother's serum contained no agglutinins acting against the baby's cells or against the Rh-positive blood of the donor. No antibody could be demonstrated by Wiener's "blocking test"[7] where the mother's serum and the baby's cells were incubated and anti-Rh serum then added. Agglutination was not inhibited by this procedure. Fourteen days after the transfusion, the mother's serum contained a weak, but definite, agglutinin against Rh-positive cells. This case is notable for several features. It is unusual for erythroblastosis to occur during the first pregnancy. The development of isoimmunity from the first transfusion is also unlikely. A transfusion reaction occurred, apparently from incompatibility to the Rh factor, although no agglutinin could be demonstrated. Wiener's blocking test failed to reveal the presence of an antibody. An anti-Rh agglutinin appeared in the serum within fourteen days after the transfusion and delivery. The free hemoglobin from the intravascular hemolysis was apparently so rapidly converted to bilirubin that none was detected in the serum immediately after the transfusion. A considerable amount of a pigment, apparently bilirubin, was excreted in the urine for many hours after hemolysis.

CASE 44-6538.—Woman, aged 30 years, Group B. The patient had never been pregnant. In 1933 she developed bronchiectasis and lobectomy was performed in the University Hospitals. The operation was complicated by empyema thoracis. During a stormy postoperative course, she received three blood transfusions (Group B) Oct. 17, 18, and 19, 1933. Each was followed by fever, but it was impossible to determine whether this was due to the infection or to the transfusions. It was the judgment of those in attendance that there had been no transfusion reactions. After operation for a gangrenous appendix, the patient received another transfusion Dec. 23, 1934, without reaction. A diaphragmatic abscess developed and was drained. Another transfusion was given without reaction Dec. 28, 1934.

During the intervening years an abscess of the lung developed at the site of the lobectomy and the patient was readmitted to the hospital with hemoptysis. On July 7, 1944, she received a transfusion of Group B blood without reaction. Another transfusion was given July 19, 1944. The patient was carefully observed during the administration of the 500 c.c. of blood because a new type of apparatus was being used. No untoward symptoms were noted during transfusion, but about twenty minutes after conclusion of the procedure, the patient had a chill and the temperature rose to 102° F. She was not considered to be seriously ill by the physician in attendance and it was not until fourteen hours later that a small amount of urine was voided which contained hemoglobin. The patient appeared apathetic and the skin was flushed. The heart rate was fast; the blood pressure measured 60 mm. of mercury, systolic, and the diastolic pressure could not be measured. Since there had been no recent loss of blood and there was vasodilatation, it was considered that the condition was primary shock. The blood pressure was restored and maintained at normal levels with subcutaneous injections of ephedrine during the day. The daily urine volume varied from 50 c.c. to 250 c.c. for the remainder of life. The values for urea nitrogen and creatinine of the blood increased rapidly. Solutions of dextrose, saline, and sodium lactate were administered parenterally. The renal pelves were lavaged with warm water through the cystoscope. With continuing oliguria, decapsulation of the kidneys was performed by Dr. R. H. Flocks, of the Department of Urology, July 22, 1944. The kidneys were normal in size and the capsules stripped with ease. The operator thought that the kidneys were slightly edematous. The urinary excretion increased but little with any type of therapy. The patient became stuporous and died July 31, 1944, on the eleventh day after transfusion. The blood urea nitrogen at the time of death was 87.5 mg. per 100 c.c., and the creatinine of the blood was 14.5 mg. per cent.

Post-mortem examination was not permitted. A blood specimen taken from the patient fourteen hours after transfusion was examined. The serum was pink and spectrophotometric tests showed the pigment to be hemoglobin. No methemoglobin was present in the serum. The urine voided during the fourteen hours after transfusion exhibited in the spectrophotometer the typical bands of hemoglobin as well as those of methemoglobin. The patient's cells were Rh negative and those of the last donor were Rh positive. The recipient's serum contained an agglutinin which reacted against the donor's cells, after incubation for sixty minutes at 37° C., but did not react when the centrifuge technique without incubation was employed.

CLINICAL MANIFESTATIONS OF TRANSFUSION REACTIONS DUE TO ISOIMMUNITY

From the analysis of transfusion reactions and the case histories of patients showing isoimmunity, it is evident that transfusion reactions due to sensitivity to the Rh factor cannot be differentiated by clinical symptoms and signs from those due to other causes. In some instances, the chills and fever, resulting from the transfusion of Rh-incompatible blood, are clinically indistinguishable in severity or in course from the symptoms resulting from the injection of pyrogens of bacterial origin. There is no conclusive evidence from this study that urticarial reactions are related to isoimmunity to the Rh factor.

Clinically there seem to be slight differences between the hemolytic reactions due to the Rh factor and those caused by transfusion of blood incompatible in the ABO system. These differences are probably quantitative rather than qualitative. They are explained by the relative weakness of the anti-Rh agglutinins. The recipient who hemolyzes blood by means of an anti-Rh antibody frequently does so at a slower rate, and the total circulating free hemoglobin is not great at any one moment. The symptoms are thus slow in developing, as witness the fact that the patient who suffered the fatal reaction exhibited no signs of clinical significance until after the entire transfusion had

been concluded. From these considerations it is probable that some instances of isoimmune reactions escaped notice in this series because of complete lack of clinical symptoms. These could only be detected by laboratory procedures which would reveal failure to attain the expected blood count after transfusion or by the demonstration of increases in serum bilirubin level after transfusion. Such indications of increased rate of hemolysis, of course, are not specific for the anti-Rh antibody but would apply equally well to the increased loss of blood which had not been properly preserved.

DIFFICULTIES IN TESTING FOR RH INCOMPATIBILITIES

There are many obstacles which present difficulty in the search for incompatibilities to the Rh factor in a blood transfusion service. The first is the lack of adequate sources of Rh-typing sera. Thus far, antisera derived from laboratory animals have not proved satisfactory. Serum collected from the rare patient who develops sufficient agglutinin titer, either by repeated transfusions or by pregnancy, constitutes the only source. The problem is still further complicated by the demonstration by Wiener[8] of at least three antisera which are required to determine with certainty that cells from any source are Rh negative.

There is a disturbing lack of adequate methods of demonstrating anti-Rh antibodies in the laboratory. Although hemolysis of Rh-positive blood frequently, or perhaps always, occurs when it is transfused into a recipient possessing an anti-Rh antibody, no one has been able to demonstrate hemolysis in the test tube. Where isoimmunity to the Rh factor probably exists, no agglutinin can be demonstrated in many instances by the methods now in use.

It is frequently necessary to work with weak anti-Rh agglutinins in cross-matching blood. Difficulty is occasionally encountered in determining whether significant agglutination has occurred. This is particularly true in dealing with preserved blood. During storage the erythrocytes sediment and sometimes adhere so that some agitation of the suspension is necessary to separate them. On the other hand, it requires little shaking to break up the agglutination caused by the anti-Rh serum. The interpretation of the finding of small clumps of cells in the erythrocyte-serum suspension is difficult. The differentiation between rouleau formation and true agglutination offers difficulties which are aggravated when preserved blood is involved. The erythrocytes during storage frequently lose their biconcave, discoid shapes so that when rouleaux form, they appear as clumps rather than as stacks of coins. The usual method of distinguishing these from clumps due to the presence of the anti-A or anti-B agglutinins is to dilute the cell-serum suspension with saline and agitate, the rouleaux being broken up readily by this procedure. This maneuver, however, also breaks up the clumps formed by the anti-Rh serum, especially when the titer is weak.

The time required for incubation of cell-serum suspensions for the demonstration of the anti-Rh agglutinin (from thirty to sixty minutes) imposes a serious delay if the transfusion is required as emergency treatment. In view of the incidence of isoimmunity to the Rh factor presented in this paper, it is the practice of staff members of the Blood Transfusion Service to forego the

incubation of cell suspensions preliminary to urgent transfusions. The delay is considered more dangerous than the possibility of a reaction from Rh-incompatible blood.

SUMMARY AND CONCLUSIONS

1. A series of 5,386 consecutive blood transfusions was studied. Multiple transfusions were given to recipients without regard to Rh type or to obstetric history of female recipients. Transfusion reactions of all types were studied for evidence of isoimmunization with the Rh factor.

2. The incidence of transfusion reactions of all types in the series was 3.4 ± 0.2 per cent. In 3,132 transfusions given to 1,112 males, the incidence of reactions was 3.1 ± 0.2 per cent. In 1,004 female recipients of all ages, the incidence of reactions was 3.9 ± 0.4 per cent in 2,254 transfusions. This is considered to be a statistically significant difference in the sexes which may possibly be explained by isoimmunity to the Rh factor developed during pregnancy.

3. In the 186 transfusion reactions of all types, only six were found which could be attributed to isoimmunity to the Rh factor, an incidence of 0.1 per cent in the 5,386 transfusions. Isoimmunity was attributed to multiple transfusions in four instances and to pregnancy in two. There was one fatality from transfusion of Rh-positive blood into a recipient who had been sensitized by multiple transfusions.

4. There were 1,717 recipients receiving from one to three transfusions. In this group, none was sufficiently sensitized to the Rh factor to give clinically significant reactions. It is estimated that of the 399 recipients receiving four or more transfusions, approximately sixty were Rh negative. Only four, or 6.6 per cent, of these were immunized to the Rh factor by multiple blood transfusions.

5. The case histories of the six recipients with isoimmune reactions are reported. Preliminary cross-matching for the anti-Rh agglutinin would not have prevented all of the reactions. A series of increasingly severe reactions in a recipient cannot be depended upon as adequate warning of developing sensitivity to the Rh factor.

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STUDIES OF SICKLE-CELL FORMATION IN NORMAL SALINE, PLASMA, AND SERA WITH CARBONIC ANHYDRASE INHIBITORS

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TO DATE, no reports have been made upon the presence, or absence, of the enzyme carbonic anhydrase in relation to the phenomenon of sickling erythrocytes. Also, considerable discrepancy exists among various reports[1-4] concerning the ability of susceptible erythrocytes to sickle when washed with normal saline and resuspended in normal saline, plasma, or sera. We desire to report controlled studies upon sickle-cell formation under the above circumstances, studies on the role of carbonic anhydrase in sickle-cell formation, and the effect of its inhibition.

MATERIAL

The blood for these studies was obtained from British West Indian or Panamanian Negroes admitted to this hospital for a variety of complaints not related to the sickling phenomenon. Blood from one outpatient with active sickle-cell anemia in an asymptomatic period was also used. Sickle-cell preparations were made in all cases by using No. 1 cover slips and sealing the edges with petrolatum. The preparations were checked immediately and in twenty-four hours. All blood used showed at least 80 per cent sickled forms in twenty-four hours at room temperature (28° C.).

I. EFFECT OF NORMAL SALINE, PLASMA, AND SERA SUSPENSION MEDIUMS ON SICKLE-CELL FORMATION

Material and Procedure.—Blood from fifteen patients with sicklemia and one with active sickle-cell anemia was available for this study. The blood specimens were, where desired, defibrinated in the usual manner and separated into cells and sera by centrifuging and resuspending the cells in normal saline (0.85 per cent). For other uses 10 c.c. of blood were placed directly into a 30 c.c. volume test tube containing 17 mg. (±) of potassium oxalate. (A one-half saturated solution of potassium oxalate was used; 0.1 c.c. of this solution was placed in the bottom of each tube and evaporated. By analysis, this has been found to leave 17 mg. [±] of potassium oxalate in each tube.) The oxalated blood was centrifuged and the plasma removed; then the packed erythrocytes were washed in the following manner. Conical 15 c.c. centrifuge tubes were used and 0.5 c.c. of packed erythrocytes and 14 c.c. of normal saline were thoroughly mixed and then centrifuged for four minutes at 2,000 r.p.m.; the supernatant saline was poured off and the packed erythrocytes were resuspended in saline and the process repeated the number of times indicated. Following the last centrifugation the supernatant saline was removed and the volume of packed erythrocytes was made up to 1 c.c. with either normal saline, plasma, or serum as desired. From these suspensions, petrolatum-sealed cover-slip prepara-

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tions were made and examined immediately and in twenty-four hours. The blood from the patient with active sickle-cell anemia was the only one which showed sickled forms (15 per cent) immediately after the sealed preparation was made.

TABLE I

PERCENTAGE SICKLING IN TWENTY-FOUR HOURS WHEN WASHED SUSCEPTIBLE ERYTHROCYTES ARE SUSPENDED IN SALINE, SAME, AND OTHER PLASMA AND SERA

TIMES WASHED	SUSPENDED IN	CASE															
		1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
5	Normal saline	100	90	80	90	95	80	80	95	90	95	100	90	100	95	100	90
10	Normal saline	–	–	–	–	–	–	20	30	50	–	25	20	30	35	40	20
20	Normal saline	–	–	–	–	–	–	–	–	–	–	Neg	Neg	5	Neg	Neg	Neg
5	Same plasma							80	90	95	–	100	90	100	95	95	90
5	Same serum							80	95	95	–	95	85	95	95	95	85
10	Same plasma							30	40	30	–	35	25	40	35	40	30
10	Same serum							25	35	35	–	40	25	35	40	35	35
5	Other plasma							75	95	90	–	90	90	95	90	95	95
5	Other sera							70	90	90	–	95	85	90	85	95	90
10	Other plasma							20	20	25	–	30	20	30	30	20	15
10	Other sera							30	15	20	–	30	10	25	10	25	20
Control untreated blood		100	90	80	90	90	80	85	100	100	90	100	90	100	100	100	90

*Active sickle-cell anemia.

Results.—From Table I it is seen that washing susceptible erythrocytes five times with normal saline has no effect upon their ability to sickle in twenty-four hours when they are resuspended in normal saline. Washing the erythrocytes ten times reduces their ability to sickle from 50 to 75 per cent, while washing twenty-times almost always removes their ability to assume sickled forms in normal saline.

In the experiments using the patients' own plasma and serum for suspending the cells after washings, five washings had little or no effect in preventing sickle formation, while washing ten times reduced the ability of the cells to assume sickled shapes from 60 to 70 per cent.

In experiments using plasma and sera from normal white and Negro persons of the same blood groups as the cases of sicklemia being studied, washing the erythrocytes five times before suspending in the plasma or sera had little or no effect, while washing ten times reduced the ability of the cells to assume sickled shapes from 45 to 75 per cent. The implications of these findings will be discussed later.

II. EXPERIMENTS ON OXYGENATED BLOOD WITH SUBSTANCES KNOWN TO BE CARBONIC ANHYDRASE INHIBITORS

Material and Procedure.—Carbonic anhydrase, an enzyme which catalyzes both phases of the reversible reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$, is present in all mammalian erythrocytes. Its presence accelerates the reaction above so that the exchange of gases can occur under normal conditions during the passage of blood through the pulmonary circulation. No studies have been reported upon

TABLE II

THE EFFECT OF CARBONIC ANHYDRASE-INHIBITING COMPOUNDS ON SICKLE-CELL FORMATION IN OXYGENATED BLOOD

	EXAMINED	CASE											
		1*	6	7	8	9	10	11	12	13	14	15	16
Sulfanilamide (1:1,000)	Immediately	–	Neg.	Neg.	–	Neg.	Neg.	–	–	–	–	Neg.	Neg.
	In 24 hours	–	40†	55	–	100	60	–	–	–	–	35	40
Ammonium thiocyanate (1:1,000)	Immediately	–	–	–	–	Neg.	–	Neg.	Neg.	–	–	Neg.	–
	In 24 hours	–	–	–	–	100	20	40	80	–	–	40	–
Zinc acetate (1:1,000)	Immediately	12	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
	In 24 hours	12	1	Neg.	1	1	1	Neg.	Neg.	1	Neg.	Neg.	Neg.
Sodium cyanide (1:1,000)	Immediately	10	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
	In 24 hours	10	Neg.	Neg.	Neg.	1	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Sodium acetate (1:1,000)	Immediately	11	–	Neg.	–	–	–	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
	In 24 hours	100	–	90	–	–	–	100	90	100	95	100	90
Zinc sulfate (1:1,000)	Immediately	10	–	Neg.	–	–	–	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
	In 24 hours	10	–	Neg.	–	–	–	1	4	Neg.	1	Neg.	Neg.

*Active sickle-cell anemia.
†Percentage sickled forms.

the presence of, or the relation between, carbonic anhydrase and the sickling of erythrocytes.

Sulfanilamide and zinc salts,[5] ammonium thiocyanate,[6] and cyanide compounds[7] have been shown to inhibit or destroy the action of carbonic anhydrase, and these substances were tested against the blood of sicklemic patients and of one with active sickle-cell anemia. The oxalated bloods were thoroughly oxygenated and normal saline solutions of the various test compounds were added to make final 1:1,000 dilutions in the blood. They were thoroughly mixed, then petrolatum-sealed cover-slip preparations were made immediately. These preparations were examined at once, in twenty-four hours, and in forty-eight hours. The twenty-four and forty-eight hour results were similar and only the twenty-four hour figures are given.

Results.—In Table II are the figures representing the percentage of sickled forms present. Sulfanilamide and ammonium thiocyanate were inconstant and incomplete in their effects. Zinc salts and the cyanide radical were complete in their effect, preventing the formation of sickle forms beyond the numbers already present, as in Case 1 of active sickle-cell anemia. The sickled forms in this case could not be changed to discoid forms by passing pure oxygen over the blood for ten minutes before starting the experiments. Sodium acetate was used as a control since the zinc salt used was an acetate and the cyanide radical was combined with sodium. It showed no inhibition of the sickling phenomenon.

III. EXPERIMENTS ON CARBONATED BLOOD WITH SUBSTANCES KNOWN TO BE CARBONIC ANHYDRASE INHIBITORS

Blood was exposed to a pure carbon dioxide stream for fifteen minutes, then mixed with carbonic anhydrase-inhibiting agents (zinc acetate, sodium cyanide, 1:1,000), and sealed cover-slip preparations were examined immediately and in twenty-four hours. Sickling was complete when preparations were examined at these times. The cover slips were then pried off and air was admitted. In contrast to the usual preparations treated by removing the cover slips after sickling has occurred, there was no resumption of the normal discoid forms.

Likewise, fully oxygenated blood was mixed with the same inhibiting agents and cover-slip preparations were made. No sickling was present immediately, in twenty-four, or in forty-eight hours.

DISCUSSION

Murphy and Shapiro[8] studied one case of active sickle-cell anemia and felt that sickle-cell formation in normal saline suspensions was variable and was influenced by the erythrocyte concentrations. They felt that high erythrocyte concentrations metabolized the oxygen in the saline and resulted in sickling, while low erythrocyte suspensions, failing to metabolize the oxygen, did not show sickle forms. They do not indicate the number or extent of washings with which the cells were treated, and no comparisons can be drawn between their work and that reported here. Our studies were completed when their report became available and we have not attempted to check the effect of cell concentrations; however, in our experiments the cell concentrations ran as high or higher than those present in the unaltered blood from our patients.

A consideration of the percentages of sickle-cell formation in saline, plasma, and sera using saline-washed cells immediately leads to the opinion that long washings remove some substance necessary for sickling, or for the exchange of carbon dioxide and oxygen which makes sickling possible. This substance is not contained in plasma or serum, inasmuch as susceptible erythrocytes after twenty washings with normal saline do not assume sickle forms when resuspended in their original plasma or sera. The substance might be carbonic anhydrase. No determinations of carbonic anhydrase content were made on the saline washing fluid because of technical difficulties. However, Scott and Mendive[9] record a 29 per cent loss of carbonic anhydrase activity by washing erythrocytes three times with 0.9 per cent sodium chloride and then extracting with alcohol and chloroform.

From the experiments on carbonic anhydrase inhibition we feel that the evidence indicates that carbonic anhydrase is probably present in normal, active amounts in the erythrocytes of people with the sickling phenomenon and does not enter into the phenomenon of sickling beyond its normal function, namely, facilitating the exchange of carbon dioxide and oxygen. Inasmuch as sulfanilamide and ammonium thiocyanate gave incomplete and inconstant inhibition of the carbonic anhydrase activity, it is possible that some other mechanism may be involved.

SUMMARY

1. Erythrocytes capable of assuming sickled shapes will do so when resuspended in normal saline, their original plasma or serum, or in other compatible plasma or serum, providing they have not been washed more than five times in normal saline.

2. Multiple washings of the erythrocytes remove some substance, possibly carbonic anhydrase, which is necessary for the phenomenon of sickling.

3. Zinc acetate or sodium cyanide in concentrations of 1:1,000, when added to oxygenated blood, prohibits sickle-cell formation.

4. Zinc acetate or sodium cyanide in concentrations of 1:1,000, when added to carbonated blood, does not reverse the sickled forms and they do not resume discoid shapes when exposed to air or oxygen.

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NONSPECIFIC COMPLEMENT-FIXING ANTIGEN IN EMBRYONIC EGG TISSUES

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COMPLEMENT-FIXING antigens prepared from embryonated chick eggs are being used extensively for the diagnosis of virus and rickettsial diseases. Bengtson[1, 2] prepared antigens for "Q" fever and murine typhus from infected yolk sac cultures, while McKee, Rake, and Shaffer[3] prepared antigens for lymphogranuloma venereum from the same tissue. Reynolds and Pollard[4] employed commercial typhus vaccine as a complement-fixing antigen for typhus fever. Plotz[5] recommended the use of purified rickettsial antigens for the diagnosis and differentiation of epidemic and murine typhus, and Smadel, Wertman, and Reagan[6] employed the same principle in the preparation of a psittacosis antigen. Yanamura and Meyer[7] prepared a psittacosis antigen from chick embryo cells grown on a semisolid medium used by Zinsser, Plotz, and Enders[8] for the cultivation of rickettsiae, while Plotz and Wertman[9] prepared a Rocky Mountain spotted fever rickettsial antigen from infected chick embryo cells grown on the same medium.

The fact that normal tissues contain antigen that reacts with Wassermann-positive sera is well known. Alcoholic extracts of infected and normal tissue have been described as Wassermann antigens by Detre[10] and Landsteiner, Müller, and Potzl.[11] Mazzini[12] described a microscopic flocculation test for syphilis in which the antigen was prepared from beef heart and egg yolk. This antigen contains an ether-insoluble, alcohol-soluble lipoid which reacts with the syphilitic antibody. Guggenheim[13] found that the injection of egg yolk caused the formation of antibodies of the Wassermann type in rabbits.

Since crude egg antigens are being used extensively in the complement fixation tests, it was thought advisable to study more thoroughly the nature of this nonspecific complement-fixing phenomenon.

In the several techniques advocated for the performance of complement fixation tests, the time of primary incubation and temperature has varied. Bronfenbrenner and Schlesinger[15] found that short periods of primary incubation at 37° C. were sufficient to permit the more active antigens and antibodies to unite and could be used for presumptive elimination of strongly positive sera. They stated that icebox incubation for from eight to ten hours, while increasing the degree of fixation, may give nonspecific reactions in that even traces of secondary antigens and their antibodies may cause fixation of complement. Kolmer, Rule, and Yagle[14] have similarly shown that a prolonged period of primary incubation at icebox temperature increases the fixation of complement.

Plotz[5] and Plotz and Wertman,[9] recognizing the possible danger of nonspecific reactions with secondary antigens at 8° C. overnight, yet wishing to use the more sensitive technique, advocated the use of highly purified antigens

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from which such nonspecific substances have been removed. With the use of such materials it has been found that the more sensitive technique of overnight fixation at from 4 to 8° C. yields much higher titers without interference of nonspecific reactions with syphilitic serum.

Because of the possibility that persons unfamiliar with the details of the preparation of these antigens might attempt to interchange the recommended techniques of primary incubation, it was decided to study the degree of nonspecific complement fixation with the use of normal egg antigens as well as with crude rickettsial antigens prepared from infected eggs when primary incubation of one hour at 37.5° C. and of eighteen hours at from 4 to 8° C. was employed.

Methods.—Complement titrations were carried out with varying quantities of a 1:30 dilution of commercial lyophilized guinea pig serum; that is, 0.08 c.c. to 0.26 c.c. with increments of 0.02 c.c. One exact unit was defined as the minimal amount of complement in which complete hemolysis of the sensitized sheep cells had occurred. A full unit was that amount in the second tube giving complete hemolysis or 0.02 c.c. greater than the exact unit. Two full units of complement in a volume of 0.5 c.c. were employed throughout the study. In order to establish whether any complement activity was lost, other than by specific fixation, parallel titrations of ½ unit, 1 unit, 1½ units, and 2 units were run for each period of primary incubation.

In the antigen titration, 0.25 c.c. of serum dilution, 0.25 c.c. of antigen dilution, and 0.5 c.c. of complement (containing 2 full units) were mixed for the primary incubation. After incubation, a suspension of sensitized red blood cells, which consisted of equal amounts of amboceptor (3) units and sheep erythrocytes (3 per cent), was added in 0.5 c.c. volume to each tube. The total volume in each tube was 1.5 c.c. Secondary incubation for thirty minutes at 37.5° C. was then employed. In reading the tests, a 4 plus (no hemolysis of the sensitized cells), and a 3 plus (25 per cent hemolysis of the cells) were taken as end points. A 2 plus (50 per cent hemolysis of the cells) or less was considered as not significant. Primary incubation, which was the only variable, was carried out for one hour at 37.5° C., four hours at from 4 to 8° C., and eighteen hours at from 4 to 8° C. The usual controls for the complement fixation technique were incorporated in each protocol.

Antigens were prepared from normal yolk sacs and embryos of developing hen eggs at the ages usually employed in preparing rickettsial and virus complement-fixing antigens; that is, 10- and 11-day-old developing chick embryos. The yolk sacs or embryos were shaken with glass beads and 10 per cent suspensions in saline were prepared. The suspensions were centrifuged at 1,500 r.p.m. for thirty minutes in an International Centrifuge, Type SB to remove the large particles, and the supernatant fluid from each suspension was employed as antigen.

The same pools of human sera from syphilitic and nonsyphilitic individuals were used throughout this study. The pool of sera from syphilitic persons was markedly positive with both Kahn and Kolmer techniques.*

Effect of Primary Incubation on Normal Egg Antigens.—Various fractions of the prepared normal egg antigens were titrated against syphilitic and non-

*Pooled serum from syphilitic and non-syphilitic individuals supplied by the Division of Serology, Army Medical School.

syphilitic sera. Twofold dilutions of antigen were tested at varying primary incubations against twofold dilutions of serum.

In the first test, normal 10 per cent yolk sac and normal 10 per cent embryo suspensions were studied (Table I). Since the nonsyphilitic sera tested at 1/5 and 1/10 dilutions were completely negative against all dilutions of antigen at the three periods of primary incubation, only the 1/5 dilution of these sera is included in the tables.

TABLE I

EFFECT OF PRIMARY INCUBATION ON REACTION OF SYPHILITIC SERA AND NORMAL EGG ANTIGENS

SERUM	SERUM DILUTION	10 PER CENT NORMAL YOLK SAC SUSPENSION* TEST INCUBATED AT THE FOLLOWING			10 PER CENT NORMAL EMBRYO SUSPENSION* TEST INCUBATED AT THE FOLLOWING		
		1 HR. AT 37.5° C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.	1 HR. AT 37.5° C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.
Nonsyphilitic pool	1/5	0	0	0	0	0	0
Syphilitic pool	1/5	1/16	1/16	1/128	1/8	1/8	1/64
Syphilitic pool	1/10	1/8	1/8	1/64	1/8	1/8	1/32
Syphilitic pool	1/20	0	0	1/32	0	0	1/8
Syphilitic pool	1/40	0	0	1/16	0	0	1/4
Syphilitic pool	1/80	0	0	1/16	0	0	0

*Twofold dilutions of antigen titrated.

In Table I it is demonstrated that both the normal yolk sac antigen and the embryo antigen are capable of fixing complement in the presence of syphilitic sera. It also illustrates that greater fixation results when the more sensitive technique of eighteen hours' incubation in the cold is employed.

It was then of interest to determine whether this nonspecific agent could be removed by ether extraction and further processing. The antigens were therefore extracted with ethyl ether, and the resulting emulsion and excess ether were discarded. The aqueous fractions were then titrated as before with the use of the same procedure (Table II).

TABLE II

REACTION OF SYPHILITIC SERA AND ETHER-EXTRACTED NORMAL EGG ANTIGENS

SERUM	SERUM DILUTION	10 PER CENT NORMAL YOLK SAC, ETHER-EXTRACTED* TEST INCUBATED AT THE FOLLOWING			10 PER CENT NORMAL EMBRYO, ETHER-EXTRACTED* TEST INCUBATED AT THE FOLLOWING		
		1 HR. AT 37.5° C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.	1 HR. AT 37.5° C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.
Nonsyphilitic pool	1/5	0	0	0	0	0	0
Syphilitic pool	1/5	1/4	1/4	1/64	1/4	1/8	1/32
Syphilitic pool	1/10	<1/4†	<1/4	1/32	<1/4	1/4	1/16
Syphilitic pool	1/20	0	0	1/8	0	0	1/8
Syphilitic pool	1/40	0	0	1/4	0	0	0
Syphilitic pool	1/80	0	0	0	0	0	0

*Twofold dilutions of antigen titrated.
†<¼ indicates a 1 plus or 2 plus reaction at that dilution.

From the results shown in Table II, it is noted that while the ether extraction removed approximately one-half of the reacting antigen from normal yolk sac and normal embryo antigens, a considerable amount of antigenic material

still remained which was more active when the more sensitive technique of primary incubation for eighteen hours at from 4 to 8° C. was used.

Ether-extracted normal yolk sac suspensions and ether-extracted embryonic tissue were centrifuged in an angle centrifuge in the cold at 12,000 r.p.m. for one hour. The resulting sediment was resuspended to the original volume with 0.85 per cent saline solution and tested as was the supernatant fluid against the sera from syphilitic and nonsyphilitic individuals. In Table III it is shown that the antigen reacting with syphilitic sera remained in the supernatant fluid after centrifugation at 12,000 r.p.m. for one hour, while the sediments did not react even when the more sensitive technique was employed.

TABLE III

REACTION OF SYPHILITIC SERA AND ETHER-EXTRACTED NORMAL EGG ANTIGENS

SERUM	SERUM DILUTION	SEDIMENT RESUSPENDED AND TESTED AT			SUPERNATANT TESTED AT		
		1 HR. AT 37.5° C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.	1 HR. AT 37.5°. C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.
*A. 10 Per Cent Normal Yolk Sac, Ether-Extracted, 12,000 r.p.m. for One Hour**							
Nonsyphilitic pool	1/5	0	0	0	0	0	0
Syphilitic pool	1/5	0	0	0	1/4	1/4	1/64
Syphilitic pool	1/10	0	0	0	1/4	1/4	1/32
Syphilitic pool	1/20	0	0	0	0	1/2	1/8
Syphilitic pool	1/40	0	0	0	0	0	0
Syphilitic pool	1/80	0	0	0	0	0	0
*B. 10 Per Cent Normal Embryo, Ether-Extracted, 12,000 r.p.m. for One Hour**							
Nonsyphilitic pool	1/5	0	0	0	0	0	0
Syphilitic pool	1/5	0	0	0	1/4	1/4	1/32
Syphilitic pool	1/10	0	0	0	1/4	1/4	1/16
Syphilitic pool	1/20	0	0	0	0	0	1/8
Syphilitic pool	1/40	0	0	0	0	0	1/4
Syphilitic pool	1/80	0	0	0	0	0	0

*Twofold dilutions of antigen tested.

Removal of Syphilitic Antigen by Absolute Alcohol Extraction.—Since Mazzini[12] has described a flocculating antigen prepared from absolute alcohol extracted beef heart and egg yolk for the diagnosis of syphilis, it seemed advisable to attempt the removal of the syphilitic-reacting substance from embryonic egg antigens by this method. A 10 per cent suspension of normal yolk sac that reacted with syphilitic sera was therefore extracted with absolute alcohol. The titration of the antigen before and after the treatment is given in Table IV.

TABLE IV

10 PER CENT NORMAL YOLK SAC SUSPENSION—PRIMARY INCUBATION EIGHTEEN HOURS AT FROM 4 TO 8° C.

SERUM	DILUTION	BEFORE EXTRACTION WITH ALCOHOL	AFTER EXTRACTION WITH ALCOHOL
Nonsyphilitic pool	1/5	0	0
Syphilitic pool	1/5	1/64	0
Syphilitic pool	1/10	1/32	0
Syphilitic pool	1/20	1/8	0
Syphilitic pool	1/40	1/4	0
Syphilitic pool	1/80	0	0

In Table IV it is indicated that the reacting antigen was removed by the absolute alcohol extraction. Moreover, the alcoholic extract behaved like lipoid antigen for syphilis and gave positive reactions with syphilitic sera.

When it was found that the nonspecific antigenic material was removed by alcohol extraction, it was necessary to determine the effect of alcohol on the antigenicity of a suspension of concentrated rickettsiae. A suspension of epidemic rickettsiae was therefore extracted with alcohol and titrated against 4 antibody units of a homologous convalescent serum. The alcohol-treated antigen was no longer active although the original material was antigenic at a dilution of 1/120.

Specific and Nonspecific Activity of Crude Rickettsial Egg Antigens.—Since Bengtson[1, 2] employed yolk sac antigens which had been purified by only one centrifugation and resuspension and since Reynolds and Pollard[4] recommended the use of commercial typhus vaccine as a complement-fixating antigen, it was considered of interest to test these antigens with syphilitic and nonsyphilitic sera, using primary incubation of both one hour at 37.5° C. and eighteen hours at from 4 to 8° C. The results obtained are indicated in Table V.

TABLE V

SPECIFIC AND NONSPECIFIC TITER OF CRUDE RICKETTSIAL EGG ANTIGENS

SERUM	SERUM DILUTION	COMMERCIAL TYPHUS VACCINE		CRUDE TYPE TYPHUS ANTIGEN	
		1 HR. AT 37.5° C.	18 HR. AT 4 TO 8° C.	1 HR. AT 37.5° C.	18 HR. AT 4 TO 8° C.
Epidemic typhus	(4 units)	1/16	1/64	1/32	>1/128
Nonsyphilitic pool	1/5	0	0	0	0
Syphilitic pool	1/5	0	1/128	0	1/128
Syphilitic pool	1/10	0	1/64	0	1/128
Syphilitic pool	1/20	0	<1/64	0	1/64
Syphilitic pool	1/40	0	1/32	0	1/32
Syphilitic pool	1/80	0	1/16	0	1/8

These results indicate that while the antigens tested did not give positive reactions with the pool of syphilitic sera employing the technique of a short period of incubation at 37.5° C. recommended by the authors, they nevertheless gave a considerable reaction with the same sera when tested by the more sensitive method of eighteen hours at from 4 to 8° C. Likewise, the degree of nonspecific reaction of these antigens was well within the range of the specific titer with homologous antiserum and indicates that in serum titrations false positive fixation with the sera from syphilitic individuals would be obtained. In fact, when the sera from ten syphilitic individuals that were Kahn positive were tested with these antigens at eighteen hours at from 4 to 8° C., eight gave fixation of complement at a serum dilution ranging from 1/10 to 1/80, and two were negative at 1/10 dilution.

The Absence of Nonspecific Reacting Substances in Rickettsial and Virus Antigens Prepared From Egg Material by Repeated Centrifugation and Resuspension.—As was indicated previously, Plotz and I succeeded in eliminating nonspecific Wassermann type antigens by repeated centrifugation and washing of the spotted fever rickettsial suspensions. It was thought advisable at this time to recheck the activity of several of the purified antigens used at the Army Medical School against their homologous antisera as well as against the syph-

ilitic pool, using the various indicated periods of primary incubation. For this purpose purified antigens for epidemic typhus,[5] murine typhus,[5] and psittacosis[6] were titrated against 4 units of homologous antibody as well as against the positive and negative syphilitic pools used in a dilution of 1/5 and 1/10.

The results obtained are demonstrated in Table VI.

In these titrations, 2 full units of complement remained in each complement titration control with each period of primary incubation. It is evident from Table VI that a primary incubation of eighteen hours at from 4 to 8° C. is the more sensitive test; that is, complement is bound at a higher dilution of antigen when tested with a constant amount of antibody. Syphilitic sera, on the other hand, did not fix complement with these antigens at from 4 to 8° C.

TABLE VI

SPECIFIC ANTIGEN TITERS AT VARYING PRIMARY INCUBATIONS

PURIFIED ANTIGENS	ANTISERUM	HIGHEST DILUTION OF ANTIGEN FIXING COMPLEMENT AT		
		1 HR. AT 37.5° C.	4 HR. AT 4 TO 8° C.	18 HR. AT 4 TO 8° C.
Epidemic typhus	Epidemic (4 units)	1/50	1/60	1/120
	Syphilitic pool 1/5	0	0	0
Murine typhus	Murine (4 units)	1/50	1/60	1/160
	Syphilitic pool 1/5	0	0	0
Psittacosis	Psittacosis (4 units)	1/8	Not done	1/24
	Syphilitic pool 1/5	0	Not done	0

DISCUSSION

This study emphasizes certain points which must be taken into consideration when employing complement-fixing antigens prepared from embryonic egg tissues. When relatively crude antigens are used, as suggested by Bengtson[1, 2] and Reynolds and Pollard,[4] the technique of eighteen hours' incubation at from 4 to 8° C. cannot be used because nonspecific fixation of complement occurs with sera from syphilitic individuals. While the technique of one hour at 37.5° C. decreases this nonspecific reaction with syphilitic sera, the specific reaction is, at the same time, less sensitive in that lower antigen titers are obtained. With purified washed antigens, on the other hand, the sensitive technique of eighteen hours' incubation, at from 4 to 8° C., can be used without nonspecific reactions with syphilitic sera. It is for this reason that only purified rickettsial and psittacosis-lymphogranuloma venereum antigens are used in the Division of Virus and Rickettsial Diseases, Army Medical School.

SUMMARY

1. Normal yolk sac and normal embryo tissues contain an antigen which reacts with syphilitic sera. This reaction is more marked with a primary incubation of eighteen hours at from 4 to 8° C. than with a primary incubation of one hour at 37.5° C.

2. This nonspecific antigen is reduced approximately 50 per cent by ether extraction and is completely removed by extraction with absolute ethyl alcohol.

3. A simple centrifugation and resuspension of the tissue sediment reduces the nonspecific activity, while repeated washing and centrifugation completely eliminates it.

4. Crude rickettsial antigens that have undergone limited processing contain this nonspecific antigen in low titer. While these antigens did not give false positives with the syphilitic pool serum used at a primary incubation of one hour at 37.5° C., they did give strong false positives with the technique of eighteen hours' incubation at from 4 to 8° C. It is possible that similar false positive reactions may occur with other syphilitic sera and some antigens even when a primary incubation of one hour at 37.5° C. is used.

5. Rickettsial and elementary body antigens that have been purified by repeated washing and centrifugation no longer contain nonspecific complement-fixing antigen and may be used in tests employing the more sensitive technique of primary incubation of eighteen hours at from 4 to 8° C.

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AN ALKALINE MEDIUM AND PROCEDURE FOR THE SELECTION OF DERMATOPHYTES IN THE PRESENCE OF SAPROPHYTIC FUNGI*

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THE literature contains information showing the pathogenic fungi, that is, the dermatophytes, to be capable of growing in media of high initial pH values. Should the common saprophytic fungi be found not capable of growing in such media, it would then appear possible to apply this principle in a medium that would favor the dermatophytes over the rapidly growing saprophytic fungi.

This paper deals with the study of the growth of both dermatophytes and saprophytic fungi in the alkaline pH range and the development of a selective medium.

HISTORICAL

The effects of pH on the growth of dermatophytes and saprophytes have been studied by many workers. In 1926 Keller[1] found that the "Kaufman-Wolf fungus" developed in media of a wide pH range from 6.8 to 12.0 with the optimum being from pH 6.8 to 7.0. He also found that *Epidermophyton inguinale* developed in a pH range of from 6 to 10 and grew best in a medium of pH 7.0.

Tate[2] refers to Verujsky (1887) as finding a neutral or slightly acid medium most favorable for growth with the optimum temperature being about 33° C., using *Tricophyton tonsurans* and *Achorion schoenleinii.* Tate,[2] using both Sabouraud's and synthetic media, found wide pH ranges for growth of the dermatophytes; the limit for growth on the acid side being from 3.0 to 4.0, while it was "beyond pH 8.0" on the alkaline side, the optimum pH being about 6.0 to 7.0. He stated that all the dermatophytes were found to have an active proteolytic enzyme which acts in an alkaline medium and can hydrolyze intact proteins (casein) with the production of free amino acids. This enzyme resembles trypsin, no pepsin being found. In *Aspergillus niger* the proteolytic enzyme acts in a strongly acid medium and resembles pepsin.

In 1929 Kadisch[3] found the best growth of the dermatophytes on Sabouraud's maltose agar, on 3 per cent peptone agar, and on Grütz-glycerine agar occurred at from pH 7.2 to 7.6. He found a tendency for cultures started on the acid side to become alkaline as a result of fungal activity. He also[4] found 37° C. unfavorable for *Achorion gypseum.* Biltris[5] grew *Trichophyton gypseum* asteroides on peptone 3 per cent and agar 1.8 per cent, with the medium at initial pH values of 3.8, 6.0, and 11. No fuseaux occurred at pH 11.

Mallinckrodt-Haupt[6] found that *T. gypseum* grown in buffered solutions for several months changed an acid medium to basic and a basic solution to almost neutral. This was true also for *Achorion quinckeanum,* but it made the basic

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medium more acid (pH 6.10 to 6.92 from pH 8.0). When these fungi were started on media at pH 6.01 to 7.19 they changed the pH to the basic side of neutrality, while *Penicillium glaucum* and "Rosa Lufthefe" made the same media more acid. These differences are explained by the ability of the pathogens to break down the protein source of nitrogen.

In 1930 Talice[7] presented data which showed *Sabouraudites granulosus* (on Sabouraud's proof medium) able to grow at a minimum of pH 4 with its maximum above 9.6. Both *Penicillium citrinum* and *Rhizopus nigricans* were able to grow at pH 2.2, with their minimum pH being below this. Their maximum pH was, as with *S. granulosus*, above 9.6. The optimum of *S. granulosus* was given as pH 6 to 7.

Levin and Silvers[8] found the reaction of the fourth interdigital space of the foot to vary in different individuals from pH 6.30 to 7.85. The higher value was obtained from a case of marked dermatophytosis. Another high value of pH 7.65 was obtained, and this, too, was from an active case of dermatophytosis of the foot.

Mallinckrodt-Haupt,[9] in discussing the change in reaction which the molds produce in the growth medium, stated that true dermatophytes, with the exception of the Epidermophyton fungi, showed strong tendencies toward the formation of alkali, while most of the saprophytic Hyphomycetes produced acid.

Cerutti[10] stated that *T. gypseum* always gives an alkaline reaction during its development, while the "Achorion of Schoenlein" and the "Sporotrichum of Gougerot and Schenck" give an acid reaction at the beginning that later turns toward alkalinity.

Belisario[11] stated that "the predilection of mycological infections for the palms and soles is due to the alkaline tendency of the sweat in these areas." Where sebaceous glands are richly supplied, mycotic infections are rarely found, he states, and ". . . the hyperacidity of the sebaceous glands at puberty, through increasing the hydrogen-ion concentration of the scalp sweat, may afford a feasible explanation of the disappearance of certain small-spore ringworms at that age."

Williams[12] noted an apparent slight increase in growth of *T. gypseum* when the pH of the medium (cysteine) was raised from 5.4 to 6.6.

Peck and Rosenfeld,[13] using "Sabouraud's bouillon" unbuffered, obtained growth of *T. gypseum* in the range of pH 3.4 to pH 10.0 inclusive. No growth occurred at pH 3.0. Using McIlvaine's buffers, they obtained growth from pH 3.4 to pH 9.0. Plates poured with buffered media showed growth throughout the whole pH range (from 4 to 10) studied, but *T. gypseum* showed definite retardation at all pH values except pH 7.0 and 8.0. *E. inguinale* was retarded through the series. *T. gypseum* was found to increase the pH once growth started, and at the end of three weeks without buffer raised the pH from 5.0 to pH 7.6, whereas with buffer the pH rose to 6.5. As all of the nonbuffered solutions rose to a pH of from 7.6 to 8.0, Peck and Rosenfeld concluded that the "optimum" pH range for *T. gypseum* must lie here. They believe that the presence of a buffer, when it interferes with the production of a pH value optimum for growth, retards the growth of the organism and explains the less vigorous growth obtained with buffered solutions as compared with that obtained in nonbuffered solutions.

In media with no buffer and pH values from 4.0-8.0, Peck and Rosenfeld obtained a change to a more alkaline pH using *T. gypseum;* at pH 8.0 there was

only a slight increase in the pH value, and at pH 9.0 and 10.0 a change to a more acid reaction took place, the pH dropping to 8.08 and 7.87, respectively. With buffers the pH increased less and dropped more (except at pH 3.4), there being a sharp drop from pH 8.0, 9.0, and 10.0.

METHODS

Cultures.—The saprophytic fungi used in this study consisted of *A. niger* No. 7, *A. niger* No. 12 (isolated from worn shoes), *R. nigricans,* Penicillium species No. 8, No. 9, No. 10, and No. 11, with the latter three species being isolated from worn shoes. All three genera are the ones commonly occurring on shoes, floors, etc. The dermatophytes used were, *Epidermophyton floccosum, Tricophyton interdigitale, Tricophyton purpureum* and *T. gypseum.*

In the early part of the research a piece of colony growth was streaked over the surface of the agar or it was shaken with water and then poured with the test media. Later, suspensions of the cultures were made with water, and both streaked and poured plates were made, using the suspension as the inoculum.

Media and Incubation.—Sabouraud's maltose agar of the following composition was used throughout this study: Bacto-peptone, 10 Gm.; bacto-maltose, 40 Gm.; bacto-agar, 15 Gm.; and water, 1,000 c.c. No adjustment of the pH was made prior to sterilization at 15 pounds steam pressure for twenty minutes.

Immediately before plates were poured with this medium the pH was adjusted by the addition of approximately 1.0 N sterile NaOH to the bottles of melted agar. Adjustment to pH 10.5 was made by the use of LaMotte purple. This was later checked with a Beckman pH meter. The media were not buffered, and it is recognized that the pH was lowered merely upon standing. Thus the pH values given are the initial values only, and the term alkaline medium refers to Sabouraud's dextrose or maltose agar adjusted to an *initial* pH of 10.5.

EXPERIMENTAL

Effects of Increased Alkalinity.—As an acid pH is used to inhibit bacteria while permitting mold growth, the effects of using a low pH of 3.0 and a high pH of 8.0 on the growth of pathogenic and nonpathogenic fungi were studied. Plates of Sabouraud's maltose agar were adjusted to a pH of 3.0 with lactic acid and to pH 8.0 with sodium hydroxide and streaked with saprophytes and dermatophytes. After incubation at 34° C. for fifteen days, both *A. niger* No. 7 and Penicillium sp. No. 8 had failed to show growth at either pH. However, with three dermatophytes (*T. interdigitale, T. gypseum* and *T. purpureum*) growth occurred in three and one-half days on the pH 8.0 medium but did not occur on the pH 3.0 medium. Therefore, pH values of 8.5 and 9.0 were tested along with pH 8.0.

Since the combined effects of temperature and pH are likely to be more pronounced than either one alone, plates of Sabouraud's maltose agar adjusted to the desired pH values were streaked with various fungi and incubated at 34° C. and at 37° C. In Table I, Part A, it is indicated that the temperatures used do not influence growth (with the exception of Penicillium sp. No. 8) until the pH of the medium is raised to 9.0. At this pH, all of the molds tested, with the exception of *A. niger* No. 7, gave less growth at 37° C. than at 34° C. At 37° C.

and pH 9.0, *R. nigricans* was completely inhibited, while Penicillium sp. No. 8 took longer to initiate growth at 37° C. than it did at 34° C. regardless of the pH used. As the pH increased from 8.0 to 8.5 to 9.0 and with an incubation temperature of 34° C., it is seen that none of the dermatophytes was inhibited.

TABLE I

GROWTH OF DERMATOPHYTES AND SAPROPHYTES *Streaked* ON SABOURAUD'S MALTOSE AGAR AT VARIOUS HYDROGEN-ION CONCENTRATIONS

INCUBATION AT 34° AND 37° C.

Part A—Media Adjusted to Initial pH of 8.0, 8.5, and 9.0

CULTURES	INCUBATION TEMPERATURE ° C.	SABOURAUD'S MALTOSE AGAR OF					
		pH 8.0		pH 8.5		pH 9.0	
		DAYS INCUBATION*		DAYS INCUBATION		DAYS INCUBATION	
		4	6	4	6	4	6
T. interdigitale	34	+++	+++	+++	+++	+++	+++
	37	+++	+++	+++	+++	++	++
T. gypseum	34	+++	+++	+++	+++	+++	+++
	37	+++	+++	+++	+++	++	++
T. purpureum	34	+++	+++	+++	+++	+++	+++
	37	+++	+++	+++	+++	++	++
R. nigricans	34	+++	+++	+++	+++	+	++
	37	+++	+++	+++	+++	-	-
A. niger No. 7	34	+++	+++	+++	+++	++	++
	37	+++	+++	+++	+++	++	++
Penicillium sp. No. 8	34	+	+	+	+	++	++
	37	-	+	-	+	-	±*

-, No growth.
+, Slight growth.
++, Good growth.
+++, Very good growth.
++++, Excellent growth,
±, Growth doubtful as to presence or type (dermatophyte or saprophyte).
Blank spaces indicate plates were not read.
p, Pinpoint "colonies."
*All plates examined also after eight days and showed no change, except Penicillium sp. No. 8 was definitely + at 37°C. pH 9.0.

TABLE I

GROWTH OF DERMATOPHYTES AND SAPROPHYTES *Streaked* ON SABOURAUD'S MALTOSE AGAR AT VARIOUS HYDROGEN-ION CONCENTRATIONS

INCUBATION AT 34° AND 37° C.

Part B—Media Adjusted to Initial pH of 9.0, 9.5, and 10.5

CULTURES	INCUBATION TEMPERATURE ° C.	SABOURAUD'S MALTOSE AGAR OF											
		pH 9.0				pH 9.5				pH 10.5			
		DAYS INCUBATION				DAYS INCUBATION				DAYS INCUBATION			
		2	3	7	10	2	3	7	10	2	3	7	10
T. interdigitale	34	+++	++++	++++	++++	+++	++++	++++	++++	+++	+++		
	37	+++	++++	++++	++++	++	+++	++++	++++	+p	+	++++	++++
T. gypseum	34	+++	++++	++++	++++	+++	++++	++++	++++	+++	+++		
	37	+++	++++	++++	++++	++	+++	++++	++++	++	+++	++++	++++
T. purpureum	34	+	++	++++	++++	+	+++			+	+++	+++	
	37	+	+++	+++		+	+++	++++		+	++	++++	++++
R. nigricans	34	+	+++			-	-	+		-	-	-	-
	37	++	+++	+++		-	-	+	++	-	-	+	
A. niger No. 7	34	+++	+++			+	+	++		-	+	++	
	37	++	++	++		++	++	++		-	±	+++	
Penicillium sp. No. 8	34	++	++	+++	++++	++	++	++	++++	+	+	++	++++
	37	-	-	+		+p	+	++		-	-	++	

TABLE I

GROWTH OF DERMATOPHYTES AND SAPROPHYTES *Streaked* ON SABOURAUD'S MALTOSE AGAR AT VARIOUS HYDROGEN-ION CONCENTRATIONS

INCUBATION AT 34° AND 37° C.

Part C—Media Adjusted to Initial pH of 10.5 and 11.5

CULTURES	INCUBATION TEMPERATURE ° C.	SABOURAUD'S MALTOSE AGAR OF							
		pH 10.5				pH 11.5			
		DAYS INCUBATION				DAYS INCUBATION			
		3	4	7	11	3	4	7	11
T. interdigitale	34	++++	++++	++++	++++	++++	++++		
	37	-	+p	+	++	-	-	+	+
T. gypseum	34	++++	++++	++++	++++	++++	++++		
	37	-	+p	+	++	-	+	+	+
T. purpureum	34	+++	+++			+++	+++		
	37	-	+p	+	+++	-	-	+	+
R. nigricans	34	-	-	+++		-	-		
	37	-	-	-	-	-	-	-	++
A. niger No. 7	34	+++	+++	-		++	++		
	37	-	-	+		-	-	+	+
Penicillium sp. No. 8	34	++	++			++	++		
	37	++	++	+++		+	+	+++	+++

The saprophytic fungi, on the other hand, showed less growth at the high pH of 9.0. Penicillium sp. No. 8 did not show less growth at the high pH but showed a slight increase in growth in this particular experiment. These results indicated possible further advantages in a still more alkaline medium. The pH was increased to 9.0, 9.5, and 10.5 with plates being streaked and incubated at 34° C. and 37° C. The results obtained (Table I, Part B) show the pathogenic molds growing about as well at pH 10.5 as at pH 9.0 with 34° C. as the incubation temperature. At the same temperature and at pH 10.5, *R. nigricans* and *A. niger* No. 7 were markedly inhibited (Fig. 1). A prolonged incubation period of fourteen days showed good growth with *R. nigricans* on the pH 10.5 medium at 34° C. but showed no growth in seventeen days on the same medium at 37° C.

The inhibition of the saprophytic fungi at pH 10.5 is greater at 37° C., but the greater inhibition of the dermatophytes at this temperature than at 34° C. outweighs the beneficial effects of the higher temperature. The pH 10.5 medium showed a distinct selective action against the saprophytic fungi by inhibiting them to a much greater extent than the dermatophytes, so this experiment using Sabouraud's maltose agar adjusted to initial pH values of 9.0, 9.5, and 10.5 with incubation at 34° C. and 37° C. was repeated with the result that pH 10.5 at 34° C. again showed the best selective action.

Inasmuch as a pH of 10.5 proved effective toward attaining the desired goal of a medium capable of inhibiting the saprophytic fungi while allowing the dermatophytes to grow, it was thought that a higher pH might prove to be more effective. Plates of Sabouraud's maltose agar adjusted to pH 10.5 and 11.5 were streaked individually with saprophytic fungi and dermatophytes with incubation at 34° and 37° C. (Table I, Part C). There was no significant difference between growth at pH 10.5 and pH 11.5 at either 34° C. or 37° C. However, it can be seen that the best selection of dermatophytes over saprophytic fungi was at 34° C.

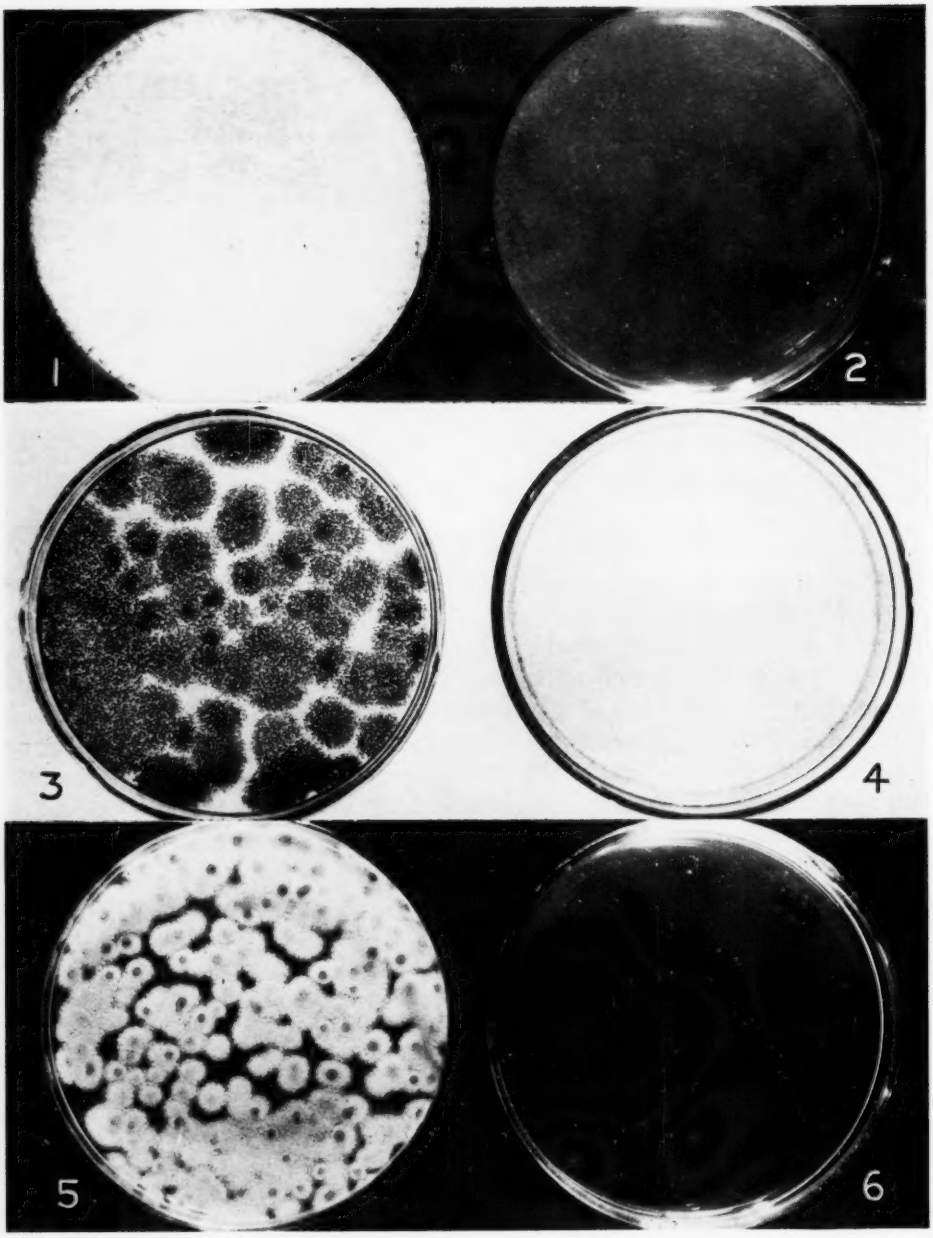

Fig. 1.—A comparison of Sabouraud's dextrose agar and the alkaline medium inoculated with pure cultures. Incubation, three days at 34°C.

Plates *1*, *3*, and *5* were poured with Sabouraud's dextrose agar, while *2*, *4*, and *6* were poured with the alkaline medium.

Plates *1* and *2* contain the same inoculum of *R. nigricans*.

Plates *3* and *4* contain the same inoculum of *A. niger* No. 7.

Plates *5* and *6* contain the same inoculum of Penicillium sp. No. 34.

Streaked vs. Poured Plates.—The test plates in all experiments so far were streaked. However, as one purpose of this study was to devise a method for the isolation of dermatophytes from shoes, a procedure based on streaking was considered inferior to that of inoculating plates with a suspension from the leather and pouring with agar. Therefore, results obtained by use of poured and streaked plates were compared.

Pure cultures of four strains of dermatophytes and seven strains of saprophytic fungi were streaked and poured individually with the alkaline medium and were incubated at 37° C. The results obtained (Table II) indicate that there was slightly better growth in the poured plates. Therefore, a comparison was made between the two techniques, using pathogenic fungi individually mixed with Penicillium sp. No. 8. The alkaline medium was streaked and poured with the mixed inocula (the same inoculum was used for both).

TABLE II

A COMPARISON OF GROWTH OF PURE CULTURES ON *Streaked* AND *Poured* PLATES USING THE ALKALINE MEDIUM (MALTOSE)

INCUBATION AT 37° C.

CULTURES	POURED							STREAKED						
	DAYS INCUBATION							DAYS INCUBATION						
	1	2	3	4	5	7	9*	1	2	3	4	5	7	9*
E. floccosum	+	+	++	+++	+++			-	-	+	+	+++	+++	
T. interdigitale	-	+	+++	++++	++++	++++	++++	-	+	+++	+++			
T. gypseum	-	+	+++	++++	++++	++++	++++	-	++	+++	++++	++++	++++	++++
T. purpureum	+	+	++	++++	++++	++++	++++	-	+	++	+++			
R. nigricans	-	-	-	-	-	+	++	-	-	-	-	-	+	++
A. niger No. 7	-	-	++	+++	++++	++++	++++	-	+	++	+++			
Penicillium sp. No. 8	+	+	++	+++				+	+	+	++	++		
Penicillium sp. No. 9	-	+	+	+	++	++	++	-	-	-	-	-	-	+++ 15 days
Penicillium sp. No. 10	-	-	-	-	+	++	++	-	-	-	-	-	++	+++
Penicillium sp. No. 11	-	-	-	-	++	++		-	-	-	+	+	++	
A. niger No. 12	++	+++	++++	++++	++++	++++	++++	-	++++	++++	++++	++++	++++	++++

*Plates examined after fifteen days showed no change other than as noted.

TABLE III

A COMPARISON BETWEEN *Streaked* AND *Poured* PLATES OF ALKALINE AGAR (MALTOSE) WITH *Mixed* CULTURES OF DERMATOPHYTES PLUS PENICILLIUM SPECIES NO. 8

INCUBATION AT 37° C.

CULTURES	TYPE OF GROWTH	STREAKED				POURED			
		DAYS INCUBATION				DAYS INCUBATION			
		1	2	3	4	1	2	3	4
T. interdigitale	P	-	±	±	+	-	±	+	+
	NP	-	±	±	++++	-	±	-	±
T. gypseum	P	-	±	±	+	-	±	+	+
	NP	-	±	±	+++	-	±	-	±
T. purpureum	P	-	±	±	+	-	±	-	+
	NP	-	±	±	+++	-	±	+	±

P, Pathogen (dermatophytes).
NP, Nonpathogen (saprophytes).

The results are given in Table III. All pathogenic fungi showed a slight growth on the streaked alkaline plates after four days' incubation, while Penicillium showed a heavy growth on the same plates. On the poured plates, growth was doubtful or poor for both the dermatophytes and Penicillium. The poured plates did not produce materially less growth of the dermatophytes compared with that obtained by use of the streaked method but did show only very slight Penicillium growth when the streaked method showed a very good growth. The advantage of the poured over the streaked plate method in inhibiting the saprophytic fungi while affecting the dermatophytes either not at all or to a less degree is definite. Using this method it is possible to inhibit Penicillium to

TABLE IV

GROWTH OF DERMATOPHYTES AND SAPROPHYTES IN PLATES *Poured* WITH SABOURAUD'S MALTOSE AGAR AT VARIOUS HYDROGEN-ION CONCENTRATIONS

INCUBATION AT 34° C.

Part A

CULTURES	SABOURAUD'S MALTOSE AGAR OF									
	pH 5.5					pH 9.5				
	DAYS INCUBATION					DAYS INCUBATION				
	3	4	5	6	7*	3	4	5	6	7*
E. floccosum	–	+	++	++	+++	+	++	+++		
T. interdigitale	++	++++	++++	++++	++++	+	++++	++++	++++	++++
T. gypseum	++	++++	++++	++++	++++	+++	++++	++++	++++	++++
T. purpureum	++	++++	++++	++++	++++	++	++++	++++	++++	++++
R. nigricans	++++	++++	++++	++++	++++	–	–	++		
A. niger No. 7	+++	+++				+++	+++	+++		
Penicillium sp. No. 8	+++	+++				++	++	++		
Penicillium sp. No. 9	++	++++	++++	++++	++++	–	+	++	++	++
Penicillium sp. No. 10	++	++++	++++	++++	++++	–	–	+	+	+
Penicillium sp. No. 11	++++	++++	++++	++++	++++	–	–	+	+	+
A. niger No. 12	++++	++++	++++	++++	++++	+++	+++	+++		

*Plates examined after twelve days showed no change, except Penicillium sp. No. 9, No. 10, and No. 11 were ++++, +++, and ++++, respectively, at pH 9.5.

TABLE IV

GROWTH OF DERMATOPHYTES AND SAPROPHYTES IN PLATES *Poured* WITH SABOURAUD'S MALTOSE AGAR AT VARIOUS HYDROGEN-ION CONCENTRATIONS

INCUBATION AT 34° C.

Part B

CULTURES	SABOURAUD'S MALTOSE AGAR OF									
	pH 10.0					pH 10.5				
	DAYS INCUBATION					DAYS INCUBATION				
	3	4	5	6	7*	3	4	5	6	7*
E. floccosum	+	+	++	+++		+	+	++	++	++
T. interdigitale	+	++	++++	++++	++++	+	+	+	++	+++
T. gypseum	++	++++	++++	++++	++++	+	+++	++++	++++	++++
T. purpureum	+	++	++			+	+	+++	+++	+++
R. nigricans	–	–	–	–	++	–	–	–	–	–
A. niger No. 7	+	+++	+++			–	+	++	++	++
Penicillium sp. No. 8	+++	+++	+++			++	++	+++	+++	+++
Penicillium sp. No. 9	+	+	+	++		–	–	–	–	–
Penicillium sp. No. 10	–	+	+	+	++	–	–	–	–	–
Penicillium sp. No. 11	–	–	–	+	+	–	–	–	–	–
A. niger No. 12	++	++++	++++	++++	++++	+	++	++++	++++	++++

*Plates examined after twelve days showed no change except as follows: at pH 10.0, *R. nigricans* was ++++, Penicillium sp. No. 10 was +++, and Penicillium sp. No. 11 was ++++; at pH 10.5, *R. nigricans* and Penicillium sp. No. 9 were ++, Penicillium No. 10 was +, and Penicillium sp. No. 11 was +++.

an appreciable extent while the dermatophytes are not so affected. Although the previous pure culture experiment (Table II) showed little difference in growth between streaked and poured plates, the latter experiment (Table III) does show the poured plate to be superior in selecting pathogenic growth from a mixture of dermatophytes with Penicillium sp. No. 8.

Using 34° C. as the temperature of incubation, a series of Sabouraud's maltose agar poured plates was prepared with the pH at 5.5 (unadjusted) and adjusted to pH 9.5, 10.0, and 10.5. The results obtained (Table IV, Parts A and B) show the efficiency of the alkaline medium (pH 10.5) in restraining growth of saprophytic fungi. In comparing the pH 5.5 with the pH 10.5 results, it is seen that the dermatophytes were inhibited by the high pH although less on extended incubation (see also Figs. 2 and 3). Nevertheless, the effect of the pH 10.5 medium upon the saprophytic fungi is more pronounced. *R. nigricans* and Penicillium sp. No. 9, No. 10, and No. 11 were completely inhibited for seven days, thus giving the pathogenic fungi ample time to produce visible growth. Six days are sufficient for pathogenic growth to appear on the alkaline medium at 34° C. On the unadjusted medium the saprophytic fungi showed a fairly rapid growth which would have overgrown any dermatophytes present. *A. niger* No. 7 was inhibited for three days at pH 10.5, showed poor growth on the fourth day and fair growth from then until the seventh day, while at the unadjusted pH of 5.5 good growth was obtained on two days' incubation. Here again, a dermatophyte would have a chance to grow in the alkaline medium but not on the unadjusted medium. Penicillium sp. No. 8 was inhibited in the alkaline medium, and although many colonies appeared, the high pH kept them from spreading over the plate. Instead, the Penicillium colonies remained small and restricted, although an occasional colony would show spreading. Therefore, any dermatophytes in the presence of this saprophytic fungus on an alkaline plate likewise would have a chance to appear, while on Sabouraud's maltose agar of unadjusted pH the rapid, spreading growth of Penicillium sp. No. 8 would greatly reduce any chance of growth by the dermatophytes. The remaining saprophytic fungus, *A. niger* No. 12, was inhibited by the 10.5 medium, for very good growth was obtained after two days' incubation at 34° C. on pH 5.5, while it took five days of incubation to produce the same results with the alkaline medium. On this alkaline medium, *A. niger* No. 12 showed no growth after two days' incubation and only fair growth after four days' incubation at 34° C. The results at pH 9.5 and 10.0 are similar to, but not as striking as, those obtained at pH 10.5. Therefore, it is seen that the use of the alkaline medium incubated at 34° C. will inhibit saprophytic fungi much more strongly than the pathogenic fungi.

It was noted, in the platings from numbers of shoes, that the predominant saprophytic fungi were Aspergillus and green Penicillium. Cultures of these two genera were combined individually with *T. gypseum* and plates poured with both unadjusted Sabouraud's maltose agar and the alkaline medium, the purpose being to test the selective action of the alkaline medium with mixed cultures and to corroborate the results and conclusions of the previous experiment. The results are given in Table V. Using *T. gypseum* mixed with *A. niger* No. 7, the Aspergillus grew rapidly and completely covered the plate of unadjusted

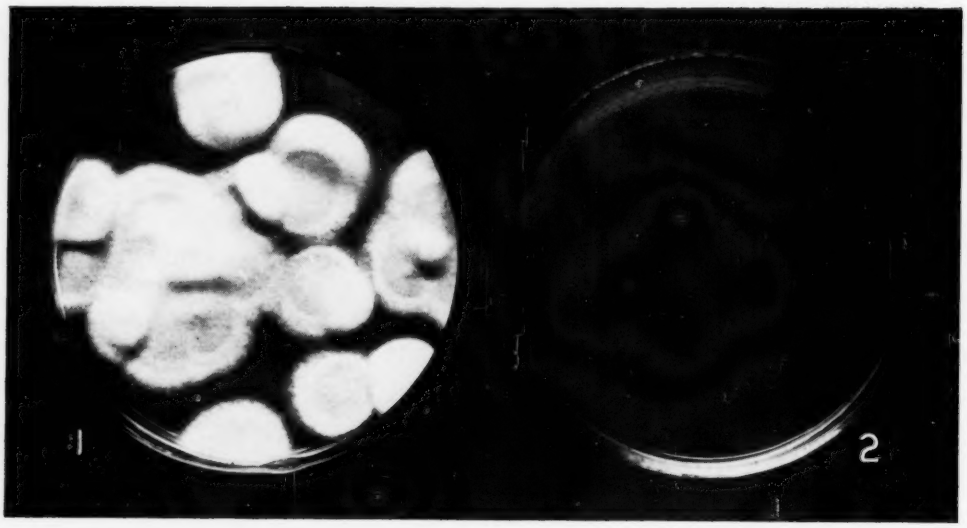

Fig. 2.

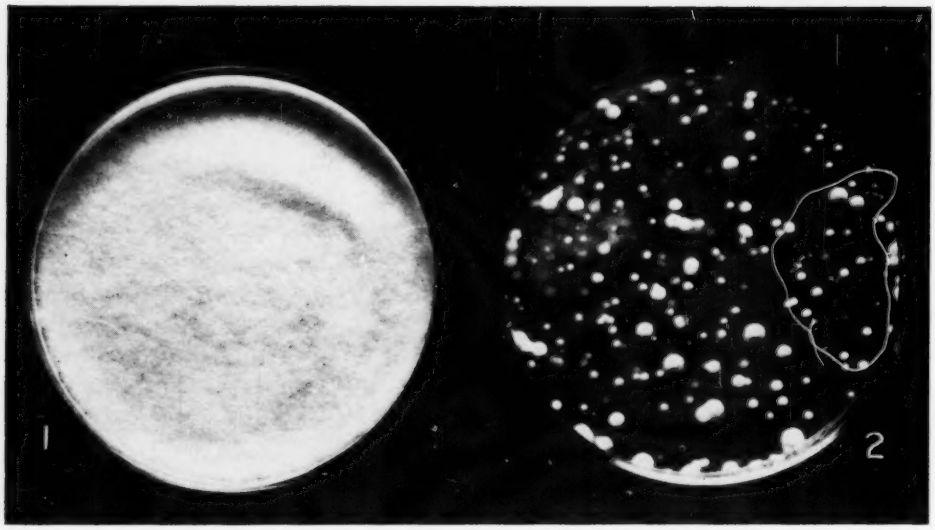

Fig. 3.

Figs. 2 and 3.—A comparison of Sabouraud's dextrose agar and the alkaline medium inoculated with a pure culture of *T. mentagrophytes* and incubated for three and for five and one-half days at 34°C., respectively.

Plates *1* were poured with Sabouraud's dextrose agar, while plates *2* were poured with the alkaline medium.

Fig. 2.—Inoculum of approximately 40. Incubation, three days.

Fig. 3.—Inoculum of approximately 40,000. Incubuation, five and one-half days.

Sabouraud's agar, and after five days' incubation no growth of the dermatophyte was visible on the medium. The same inoculum plated with the alkaline medium showed many colonies of *T. gypseum* and only a few of Aspergillus. When Penicillium sp. No. 8 was used as the saprophyte, no growth of *T. gypseum* appeared in either the alkaline or the unadjusted medium because of heavy growth by the Penicillium which covered the entire surface of the agar. It is interesting to note (Tables II and IV) that the different strains of Penicillium used

TABLE V

THE SELECTIVE ACTION OF THE ALKALINE MEDIUM UPON MIXTURES OF T. GYPSEUM PLUS A. NIGER NO. 7 AND T. GYPSEUM PLUS PENICILLIUM SP. NO. 8 (PLATES POURED)

INCUBATION, FIVE DAYS AT 34° C.

CULTURES	SABOURAUD'S MALTOSE AGAR (pH 5.5)	ALKALINE MEDIUM
T. gypseum alone	Colonies small, white, and spreading	Colonies smaller, white, and fewer in number
A. niger No. 7 alone	Spreading black growth	Colonies are separate, yellow with few black spores
Penicillium sp. No. 8 alone	Numerous green colonies	Colonies green, smaller, and less in number
T. gypseum + A. niger No. 7	Plate black with A niger; no pathogens visible	Few Aspergilli; pathogen colonies present
T. gypseum + Penicillium sp. No. 8	All green; no pathogens	All green; no pathogens

varied in their reaction on the alkaline medium. Strain No. 8, used in the experiment just described (Table V), was the one which had previously shown the best growth in this medium. Tate (see under Historical) found that the dermatophytes have a trypsinlike enzyme, while a saprophytic fungus as *A. niger* does not. As trypsin is effective in alkaline substrates, it may explain why the dermatophytes are able to grow comparatively well on pH 10.5 media, while the saprophytes are not. Also, the nature and extent of the enzyme systems may account for the variable reactions of the Penicillium strains used. On this basis, Penicillium sp. No. 8 may very well have a strong trypsinlike enzyme system and may, therefore, approach a pathogenic strain.

DISCUSSION

The study of the growth of dermatophytes and saprophytic fungi on Sabouraud's maltose agar of increasing alkaline pH concentrations has shown that the dermatophytes are capable of growing on media initially adjusted up to pH 11.5, whereas the saprophytes were largely inhibited at values above pH 9.0. The pH at which the best inhibition of saprophytes with the least comparative inhibition of dermatophytes occurred was at pH 10.5. It was found that poured plates were superior to streaked plates in inhibiting the saprophytic fungi and, therefore, in giving the best selection of dermatophytes.

A temperature of 37° C., while more effective than 34° C. in eliminating or reducing saprophytic growth, was found to be more inhibitory to the dermatophytes. Also, 34° C. is to be preferred over a fluctuating room temperature.

The experimental work also showed that an incubation period of five and one-half days at 34° C., using poured plates of the alkaline medium (Sabouraud's maltose agar of pH 10.5), gave the best results. If plates containing colonies of saprophytic fungi are incubated longer than five and one-half days, these fungi may overgrow the dermatophytes. However, plates which after five and one-half days are negative for pathogenic fungi, or on which there is no overgrowth of possible dermatophytic colonies, should be incubated for about ten days, with the plates being observed daily. Colonies of mold growth that are thought to be pathogenic should be transferred to ordinary Sabouraud's dextrose agar (or other suitable media) for purification and examination.

The alkaline medium inhibits the saprophytic fungi to a great extent. Rhizopus is usually completely inhibited, while Aspergillus does appear at times as small colonies. Penicillium grows better than the other two saprophytes, but the colonies usually remain small and restricted. The dermatophytes are inhibited on the alkaline medium and growth may not be apparent until after three days' incubation, with the colonies tending toward "fluffiness" (although granular colonies do develop). Some of the cultures, when transferred to Sabouraud's medium, regained their granular appearance.

The effectiveness of the alkaline medium in selecting dermatophytes when in the presence of saprophytic fungi may be due to the presence of a trypsin-like enzyme system in the dermatophytes but not in the saprophytic fungi (see under Historical). Such an enzyme system being effective in alkaline substrates allows the dermatophytes but not the saprophytic fungi (on the whole) to grow in the alkaline medium and relates pathogenicity to pH tolerance. The presence of such an enzyme system and the resultant ability to grow in highly alkaline substrates may well be characteristic of pathogenic fungi and bacteria* and of pathogenicity itself.

As the alkaline medium allows the selection and isolation of dermatophytes in the presence of rapidly growing saprophytic fungi, it may well be used in the isolation of dermatophytes from worn shoes, floors, shower rooms, feet, etc. Weidman[14] wrote that "several workers (personal communications) have failed in attempts to demonstrate dermatophytes in old shoes, etc.; in each case it was on account of the rank overgrowth of saprophytic molds. Even were the dermatophytes present and growing, they could not have been recognized." Bonar and Dreyer[15] were not able to cultivate dermatophytes from hair found in dressing and shower rooms and showing the presence of pathogenic fungi under microscopic examination because of "the great difficulty of isolating these comparatively slow growing organisms from such material."

Neal and Emmons[16] stated that "failure to isolate any dermatophytes from the floors of showers and locker rooms was probably due to the rapid growth of more vigorous organisms and the consequent suppression of pathogens. Probably an improvement in the technique of examination and isolation would have revealed the presence of ringworm fungi on the floors."

Further studies on the use of the alkaline medium for the isolation of dermatophytes from mixed cultures and from leather will be reported later.[17]

SUMMARY AND CONCLUSIONS

An alkaline medium with a culturing procedure which is selective for dermatophytes in the presence of saprophytic fungi has been presented. Mixed suspensions of saprophytes and dermatophytes plated in this medium produced ample growth of the dermatophyte to permit isolations.

Incubation at 34° C. of poured plates of the alkaline medium was most effective. The plates should be examined after five and one-half days' incubation, and longer periods may be satisfactory under conditions discussed in the paper.

A possible relationship between pH tolerance and pathogenicity is emphasized. The value of the procedure in the isolation of causative agents in skin

*A short report of a preliminary study on bacteria is in preparation.

infections, from worn shoes, floors, shower rooms, socks, etc., and in the purification of cultures of dermatophytes contaminated with saprophytes is suggested.

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THERAPEUTIC EFFECTIVENESS OF PENICILLIN IN TREATMENT OF VINCENT'S STOMATITIS AND ITS FAILURE TO INFLUENCE FAVORABLY CERTAIN OTHER MEDICAL CONDITIONS

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THE Surgeon General, U. S. Army, has encouraged some of the general hospitals to use penicillin in various medical diseases. Such an exploitation of this relatively new therapeutic agent has been undertaken obviously for the purpose of establishing accurate limits demarcating its value. In this hospital, an attempt has been made, therefore, to treat a variety of diseases with the drug. While the experience in general confirms that of other clinical investigators, it would seem of value to record the good therapeutic results obtained in the treatment of Vincent's stomatitis and the failure of penicillin to influence favorably a number of other medical diseases.

Forty-three patients with Vincent's stomatitis were treated with from 600,000 to 1,000,000 units of penicillin. The antibiotic was given intramuscularly in doses of 25,000 units each three hours. The average total dose was 721,000 units. All of the patients were cured. After forty-eight hours of therapy, it was usually impossible to find fusiform bacilli and spirochetes except in those in whom marked dental caries existed. In these patients, furthermore, the organisms disappeared rapidly after dental prophylaxis. It is interesting that oral temperatures frequently rose from a normal level to from 99.6 to above 101° F. during the time that penicillin was being administered. This phenomenon is unexplained and was not noted during the treatment of other diseases.

Three patients with chronic and recurring furunculosis were treated with doses of from 630,000 to 1,475,000 units. There was evidence of prompt improvement. Relapse, however, has since occurred in one patient. Penicillin, in our opinion, should be regarded only as a palliative or temporarily helpful agent in the treatment of recurring boils. It was injected for an average of eleven days.

Eleven patients with acne vulgaris were treated with doses of penicillin ranging from 2,750,000 to 3,650,000 units. While an occasional patient showed signs suggestive of improvement, none was cured nor markedly improved. Ten patients with staphylococcic dermatitis were treated with from 2 to 3 million units. Again some showed evidence of improvement but relapse was frequent; it is felt that penicillin has very little, if any, curative value in this skin disease. Penicillin was injected for an average of eighteen days.

In seven cases of asthma which were thoroughly studied and were thought to be bacterial in origin the patients were treated intensively with doses of

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from 1 to 5 million units. Various strains of streptococci and staphylococci were recovered from the paranasal sinuses of these patients. Initially there seemed to be some evidence of improvement, but it was not sustained. Penicillin was injected for an average of fourteen days.

Five patients with malaria were treated with doses varying from 2 to 5 million units of penicillin. While there was some shifting of the white blood count and a disturbance in the periodicity of the paroxysms, the therapeutic agent had no curative value. It should be added that these five patients have been treated subsequently with antimalarial agents in relatively small doses and have responded promptly. They have had no recurrences after an observation period of six months. It is conceivable that penicillin might possibly have caused them to be more responsive to antimalarial drugs.

Five patients with rheumatic fever were treated with entirely negative results. The total dose used varied from 1,400,000 to 2,600,000 units. Two patients with subacute bacterial endocarditis, proved at autopsy, received 5,600,000 units and 5,009,000 units during a period of twenty-eight and twenty-five days, respectively. Although the blood cultures became negative while penicillin was being administered, they became positive again when treatment was stopped.

One patient with disseminated lupus received 6 million units over a long period of time. On two occasions it was given for two weeks because of pneumonic complications and for an additional two weeks for the basic condition, making a total of 6 million units during a period of six weeks. The patient manifested recurrent bouts of painful swollen joints, bilaterial pleurisy, pericarditis, two attacks of streptococcic pneumonia (alpha and beta hemolytic), three attacks of jaundice, transient skin manifestations characteristic of lupus erythematosis, several bouts of unexplained diarrhea, a more or less continuous low-grade fever, weight loss, and anemia. At no time was there any evidence of clinical improvement with penicillin.

One or more patients with one of the following diseases failed to respond to the administration of penicillin: chronic pyelonephritis (given over 2 million units; aerobic and anaerobic nonhemolytic streptococcus and nonhemolytic staphylococcus isolated from urine), diphtheria; mumps; eczema; folliculitis; dermatitis herpetiformis; acute myeloid leucemia; and follicular lymphoblastoma (Brill-Symmer's disease). One patient with a peritonsillar abscess failed to improve when given penicillin until the abscess was drained surgically. Several of the patients treated with penicillin also had trichophytosis; this latter lesion showed no improvement.

As for reactions, there was noted in only three patients a mild to moderate urticaria which disappeared within a few days during penicillin therapy.

SUMMARY

Forty-three patients with Vincent's stomatitis showed prompt recovery when treated with penicillin. The failure of this chemotherapeutic agent to influence favorably a number of other medical diseases is recorded.

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EVALUATION OF TECHNIQUES USED IN THE DIAGNOSIS OF ENTEROZOIC PARASITISM IN CHILDREN

Captain Royal L. Brown

IN THIS paper is described the comparative value of several techniques used in a survey designed to determine the incidence of enterozoic parasitism in children.[1] In the 512 children between the ages of 6 weeks and 12 years included in the survey, a total of 646 specific parasites was found. The average incidence, therefore, was approximately 1.3 parasites per child. The frequency with which the different parasites were found with the combined methods was as follows: *Entamoeba histolytica*, 8.0 per cent; *Entamoeba coli*, 35.2 per cent; *Endolimax nana*, 30.5 per cent; *Dientamoeba fragilis*, 3.9 per cent; *Iodamoeba buetschlii*, 1.4 per cent; *Giardia lamblia*, 32.2 per cent; *Chilomastix mesnili*, 5.1 per cent; *Retortamonas intestinalis*, 2.3 per cent; *Trichomonas hominis*, 1.4 per cent; *Ascaris lumbricoides*, 0.6 per cent; *Enterobius vermicularis*, 4.1 per cent; *Trichuris trichiura*, 0.4 per cent; and *Taenia sp.*, 1.2 per cent.

METHODS OF STUDY AND RESULTS

Identification of the macroscopic adult helminths was possible with the unaided eye. For identification of all other parasitic forms, it was necessary to prepare the fecal specimens for microscopic examination. The techniques used and the contribution each made to the total number of parasites recognized are as follows:

1. *Wet Saline Film.*—Films prepared by placing a coverslip over a thin suspension of feces in Ringer's solution possess the advantages of being simple to prepare and of causing no morphologic distortion. In these preparations, furthermore, it is possible to observe the diagnostically characteristic motility of trophozoites and larvae. Their chief disadvantage lies in the fact that cytologic details are poorly outlined.* Eighty per cent of all parasites found were identified with this technique alone (Table I). Amoebae, however, particularly *Dientamoeba fragilis* and *Iodamoeba buetschlii*, were not well differentiated.

2. *Wet Iodine-Stained Film.*—The substitution of Lugol's iodine in the preparation of wet films brought into stained relief certain distinguishing morphologic characteristics and inclusions. Confusing plant forms, that is, blastocystis and other yeast, hair roots, and plant cells, were easily identified. Starch grains and particles (blue) and glycogen (pink) stained with iodine. The iodine, however, rapidly stopped locomotion and failed to give complete cytologic detail. About 35 per cent of the parasites which had not been identified in the saline film were differentiated in these preparations, and enough additional parasites were recognized to increase the total survey incidence by about 2 per cent.

*Diaphragm manipulation or partial dark-field illumination outlines some of the cytologic details.

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This survey was made possible by the assistance of Dr. J. H. Clark of the Pathology Department of the Philadelphia General Hospital, and Dr. D. H. Wenrich of the Zoology Laboratory of the University of Pennsylvania. The author, now in Service, made all the stool examinations.

TABLE I

COMPARATIVE IDENTIFICATION AND INCIDENCE

PARASITE SPECIES	TOTAL SPECIES	RINGER'S SALINE		LUGOL'S IODINE		IRON-ALUM-HEMATOXYLIN		CONCENTRATION			
								DE RIVAS		$ZnSO_4$	
		NO.	%	NO.	%	NO.	%	NO.	%	NO.	%
Entamoeba histolytica	41	30	70	35	85	41	100	15	37	5	100
Entamoeba coli	180	138	77	128	71	180	100	180	100	35	100
Endolimax nana	156	140	90	151	96	155	99	53	34	27	85
Dientamoeba fragilis	20	2	10	6	30	20	100	0	00	2	33
Iodamoeba buetschlii	7	0	00	6	86	7	100	0	00	3	75
Giardia lamblia	165	142	86	129	78	165	100	165	100	43	100
Chilomastix mesnili	26	24	92	16	62	25	96	2	8	3	75
Retortomonas intestinalis	12	12	100	4	33	12	100	0	00	0	00
Trichomonas hominis	7	6	86	3	43	7	100	0	00	0	00
Ascaris lumbricoides	3	2	67	2	67	2	67	3	100	3	100
Enterobius vermicularis	21	21	100	12	57	5	24	0	00	2	33
Trichuris trichiuria	2	1	50	1	50	1	50	2	100	2	100
Taenis sp.	6	6	100	3	50	2	33	2	33	2	33
Total	646	524	80	496	76	622	96	422	65	127	93

The column head No. refers to the number of parasites definitely identified by this procedure; this is represented under the column head % as per cent of total.

The iron-alum-hematoxylin slide was used to verify the identification of other films. The less than 4 per cent not identified by this slide were definitely identified on other films but could not be found on the iron-alum-hematoxylin-stained slide (concentrate or film). The actual incidence on the iron-alum-hematoxlyn without concentrate material was between 70 and 75 per cent. Nine of the twenty-one Enterobius diagnoses were made by recovery of the adult worms from the stool.

3. *Dry-Stained Slide.*—Because of the importance of nuclear cytology in identifying species of amoebae, a routine iron-alum-hematoxylin-stained slide was made on every stool and studied for at least fifteen minutes. The slide was prepared according to the method of Wenrich.[2]

Examination of the stained film made it possible to identify 96 per cent of all observed parasites and to distinguish other morphologically similar organisms and objects. It also served as a semipermanent exhibit of the organism and preserved the material for future comparison and study. On the other hand, it increased the total surveyed incidence by only 1 to 2 per cent.

4. *Concentrated Preparations.*—The de Rivas centrifugation method[3] was used routinely. Concentration by the zinc sulfate centrifugal flotation method was frequently done for comparative study.

The de Rivas centrifugation method concentrated the cysts of *Entamoeba coli* and *Giardia lamblia* and the ova of *Ascaris lumbricoides* and *Trichuris trichiura* very well, but there was some morphologic distortion. The other parasitic forms were concentrated poorly or not at all. Blastocystis and detritus were advantageously removed. The zinc sulfate centrifugal flotation was much less selective and concentrated all surveyed parasites about equally well.[4] The combined concentration methods added 11 per cent to the total number of parasites found in the survey.

DISCUSSION

The combined concentration methods permitted the finding and recognizing of the greatest number of parasites; the Ringer's saline wet film was next in this respect. The addition of Lugol's iodine to wet films killed the trophozoites but increased the number of cysts which could be identified. Preparations stained with iron-alum-hematoxylin were valuable chiefly because they provided morphologic details which aided the identification. The number of organisms

recognized on these films was unduly high (Table I) because concentrated material was stained to help the identification of cysts.

The large species of *Entamoeba histolytica* could often be identified in the simple wet films. The mixed amoebic infections and the smaller species of amoeba, however, frequently required iron-alum-hematoxylin stains for differentiation. A large per cent of *Entamoeba coli* could be identified on any of the films; iron-alum-hematoxylin was necessary in some atypical forms and mixed infections. *Endolimax nana* gave little diagnostic difficulty in the wet films except when there was mixed amoebic infection. This was also true of *Dientamoeba fragilis* and *Iodamoeba buetschlii*. Giardia and Chilomastix were easily identified in any of the films. *Trichomonas hominis* was infrequently confused with the trophozoites of Chilomastix. All ova found were easily identified in all films.

SUMMARY

1. Eighty per cent of all surveyed parasites were identified by a wet saline film; 35 per cent of the remainder were specifically diagnosed by the wet iodine-stained film. The iron-alum-hematoxylin-stained slide is an invaluable adjunct to differential diagnosis of enterozoic protozoa.

2. Routine concentration contributed 11 per cent to the total surveyed incidence.

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THE LOW TOXICITY IN ANIMALS OF BORIC ACID AS A PRESERVATIVE AGENT

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PRIOR to 1906 in the United States and to 1928 in England, boric acid was used as a food preservative at levels as high as 0.5 per cent. Wiley,[1] in his classic studies in 1904, showed that symptoms of chronic toxicity appeared in human beings when amounts of from 1 to 5 Gm. of boric acid daily were ingested with food. One-half gram daily, however, was tolerated according to Wiley for fifty days with little or no evidence of ill effect. He concluded, "On the whole, the results show that 0.5 Gm. of boric acid per day is too much for the normal man to receive regularly. On the other hand, it is evident that the normal man can receive this amount for a limited period of time without much danger of impairment of health."

Despite its checkered career as a food preservative, boric acid has continued in general use as a mild antiseptic. Some knowledge of its acute toxicity has appeared in the literature due to its accidental misuse. In 1928 McNally and Rust[2] reported deaths in six infants who were given by mistake saturated solutions containing from 3 to 6 Gm. boric acid. Peyton and Green,[3] in 1941, reported severe toxic effects but survival in a patient who received 700 c.c. of a 4 per cent solution of boric acid by hypodermoclysis. These authors suggest that the toxicity of boric acid in such large doses may be due to a displacement of chloride with a resulting upset of the chloride balance. A fall in blood chlorides during injection of boric acid was offered in support of this theory. Absence of aftereffects anywhere in the patient after receiving an almost fatal dose of boric acid was taken as evidence that the primary toxicity of the drug is functional. In 1943 Ross and Conway[4] reported death of a hospitalized boy following irrigation of an empyema cavity with saturated boric acid solution. The amount of boric acid used was heroic by any standards, about 12 liters in seventy-two hours, a total of about a pound of boric acid.

In early studies Rost[5] and Wiley[6] showed that doses of 3 Gm. of boric acid and upward were rapidly excreted in the urine to the extent of from 82 to 100 per cent. Excretion in feces and sweat was found to be small and quantitatively unimportant. Presnell and Brill,[7] in 1937, further demonstrated the rapid urinary excretion of boric acid.

Kent and McCance[8] called attention to the significant amount of boron which is an inevitable component of our daily diets. Boron is essential to the normal growth and development of several plant species and is now accepted as a constituent of all vegetable tissues. Hove and co-workers[9] found boron in all samples of milk and eggs tested, and Dodd[10] found about 0.005 per cent boric acid in several fresh fruits, with a maximum of 0.109 per cent on a dry basis.

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In balance studies, Kent and McCance[8] showed that a normal turnover from food sources was about 100 mg. of boron per week in two individuals, equal to about 80 mg. of boric acid per day. Added boric acid was quantitatively excreted in a few days.

Kahlenberg[11] and Kahlenberg and Barwesser[12] discovered the truly remarkable fact that boric acid is readily absorbed through intact human skin and may be detected in the urine within fifty-five seconds after immersion of the feet in saturated boric acid solutions. Ochsner[13] had previously shown that mere application to the skin of large compresses saturated with boric acid solution resulted in concentrations of boric acid in the urine of a few hundredths to as much as 0.1 per cent. All tissue membranes tested were readily permeable to boric acid, and this would account for its rapid excretion through the kidney.

We became interested in the toxicity of boric acid when Frost[14] found that riboflavin-boron complexes are considerably more soluble in water than is riboflavin alone. This led further to an investigation of the preservative properties of various levels of boric acid for injectable solutions containing riboflavin alone and in combination with other B complex factors. When it was found that such solutions were well preserved and well tolerated, the use of boric acid as a preservative at 0.5 per cent was extended to other experimental solutions used routinely in animal work in small amounts. Boric acid, unlike other preservative agents, such as the phenols, chlorobutanol, and benzyl alcohol, does not appear to enter into the metabolism of the animal and is excreted quantitatively as such. From this standpoint and because boron is a natural constituent of the diet, it would appear to deserve reconsideration as a preservative agent.

The following experiments are presented as evidence for the essential nontoxicity of low levels of boric acid. The preservative properties of boric acid were also investigated.

Injection Experiments With Dogs and Rats.—Long-term nutritional experiments with rats and dogs which received three weekly injections of a B complex preparation containing 0.5 per cent boric acid as a preservative were carried out as follows:

Five puppies started at from 6 to 8 weeks of age on a highly synthetic diet were supplied five B complex vitamins exclusively by injection. The injectable B complex preparation contained the following per cubic centimeter: thiamine, 1 mg.; riboflavin, 1 mg.; nicotinamide, 35 mg.; calcium pantothenate, 2 mg.; pyridoxine, 0.6 mg.; and boric acid, 5 mg. Injections were made thrice weekly at the rate of 0.23 c.c. per kilogram body weight equal to 1.15 mg. boric acid. Thus a 10 kg. dog received 11.5 mg. boric acid per injection equal to 0.5 mg. boric acid per kilogram body weight per day. Two puppies, which were both littermates of dogs receiving the B complex preparation with boric acid by injection, received an identical diet with all synthetic B complex factors added to the diet. Since these two dogs did not receive boric acid at any time, they served as controls.

Excellent tolerance of continued intramuscular injection of the above preparation over periods of from twelve to eighteen months was observed. No irritation or itching was observed to occur at the point of injection as is often seen when phenol is used as the preservative. The upper leg muscles of the two hind

legs of the dogs were injected alternately with no attempt to prepare or clean the point of injection. The dogs very soon came to take the injections as a matter of course and even welcomed the attention. There was no outward difference in the growth performance or general behavior between the injected dogs receiving boric acid and those which received their nutritional requirements entirely by mouth with no boric acid.

Similar experiments extending through two generations were carried out with rats on purified diets. Fourteen rats, seven males and seven females, were placed at weaning on a standard purified diet based on sucrose, casein, and salt mixture. These rats received by injection five synthetic B complex vitamins in the form of a preparation as described for the dog experiments. Injections were made at the rate of 0.07 c.c. per rat three times per week. This entailed an average weekly injection per rat of 1.05 mg. boric acid, equal to 0.15 mg. per day. Since the weight of the rats increased from an average of about 45 Gm. at the start of the experiment to about 217 Gm. after about fifty days, the per kilogram injection of boric ranged downward from about 3.3 to 0.7 mg. per kilogram body weight per day.

A control group of twenty-eight rats was fed an identical diet simultaneously but received all B complex vitamins orally as additions to the diet. Rats of both groups were maintained on the respective dietary regimens described through two generations. Matings were made when the rats were 75 days of age. The rate of growth, reproductive performance, average number of offspring in litters, and survival of young were about equal for the two groups as shown in Table I.

TABLE I

GROWTH AND REPRODUCTIVE PERFORMANCE OF RATS ON HIGHLY PURIFIED DIET RECEIVING BORIC ACID BY INJECTION (FROM 0.7 TO 3.3 MG. PER KILOGRAM)

GENERATION	NUMBER OF ANIMALS	AV. WT. 75TH DAY ♀	AV. WT. 75TH DAY ♂	NUMBER OF LITTERS	AV. NUMBER IN LITTERS	% YOUNG SURVIVED TO 21ST DAY	AV. WT. 21ST DAY
Group Received Boric Acid by Injection							
Parent	28 (15♀)	187	292	13	7	64	37
First	31 (23♀)	170	266	13	8	21	32
Control Group							
Parent	14 (7♀)	175	264	7	7	57	31
First	12 (8♀)	189	275	6	9	39	28

The differences in per cent survival of the young born to parent and first generation females in the two groups are not considered significant. The same applies to the differences of the average weight at the twenty-first day.

Subacute Toxicity Studies.—The object of the following experiments was to study the effects in rats and dogs of injection of large amounts of a solution containing 1.5 per cent boric acid. The solution also contained 0.1 per cent riboflavin.

Experiments in Rats: Two groups of six weanling male rats (average weights, 49 Gm.) were injected subcutaneously daily with 2 c.c. of the solution mentioned in the preceding paragraph and 2 c.c. of physiologic saline, respectively. The injections were continued for thirty days and weights were taken

at weekly intervals. The average weight increments of the groups were 106 Gm. and 108 Gm., respectively. The rate of injection of boric acid thus decreased from about 600 mg. per kilogram body weight at the start to about 180 mg. per kilogram at the end.

At the end of the period, urine tests showed the absence of sugar and a trace of albumin for the experimental animals. Blood studies made at the end of the period on three test rats and two control rats revealed no significant differences in hemoglobin, erythrocyte, or differential white blood cell counts.

Autopsies on the test rats showed no gross pathology and no irritation at the sites of injection. Hearts, adrenals, and testicles were normal. The liver showed a moderate degree of cloudy swelling and some fatty infiltration but no necrosis. The kidneys were not different in appearance from the kidneys of untreated controls. No necrosis or glomerular changes were noted.

B. *Experiments With Dogs:* Three adult dogs were injected daily intravenously with 20 c.c. of the test solution. The dogs weighed 6, 6, and 8 kg., respectively, and thus received 50, 50, and 38 mg. of boric acid per kilogram body weight, equivalent to an intake of from 2.7 to 3.5 Gm. of boric acid in terms of a 70 kg. man. Injections were continued daily for thirty days, during which time the weights of the dogs did not change significantly.

If the injections were made rapidly, the dogs became unsteady; however, they always recovered completely within a few moments and otherwise showed a normal behavior throughout the experimental period. With repetition of injections, the equilibrium disturbances disappeared.

Blood values for each dog were determined at the beginning, during, and at the end of the experiment so that the animals acted as their own controls. No significant changes in hemoglobin, erythrocyte, or differential counts were observed during the course of the experiment.

Two of the dogs were killed one day after the last injection. The urine of both dogs showed an alkaline reaction and was negative for sugar, albumin, and urobilinogen. There was no gross pathology. Histologic examination indicated a normal condition of the adrenals, spleens, hearts, intestines, and lungs. The liver showed a moderate degree of cloudy swelling. Fat stains were negative in both animals. The kidney of one dog showed some amorphous exudate in the glomerular space and in the tubules. There were some old degenerative changes in a few glomeruli. The picture was altogether not considered as presenting a severe degree of renal damage. The kidney of the second dog showed only a mild hyperemia and cloudy swelling.

Effects on Blood Pressure.—Two injections of 10 c.c. each of a 1.5 per cent boric acid solution were made intravenously into a chicken under phenobarbital anesthesia. Following each injection, there was a gradual rise of the blood pressure of about 20 mm. Hg, lasting for about three minutes. Injection of an equal volume of saline failed to cause a rise.

Injection of 20 c.c. of the solution in an anesthetized dog of 11 kg. weight, given within three minutes, had no significant effect on the blood pressure.

Effect on Estrus Cycle.—The estrus cycles of six female rats were followed for two weeks; then the rats were injected subcutaneously daily at the rate of 12 mg. of boric acid per day for twenty-one days.

Two rats showed a slightly prolonged period of diestrus at first during the injections, but the normal cycles reappeared. The other rats showed no variation from the normal during the injections.

Publman,[15] in 1939, reported that 0.5 per cent boric acid in the diet inhibited the estrus cycles of rats and that 1.0 Gm. per kilogram subcutaneously daily caused a picture of incomplete permanent estrus. Such doses are, of course, excessively high and are well above the range which Wiley[1] had previously reported to produce acute toxic symptoms in human beings. Our results show, on the other hand, that daily doses as high as from 60 to 70 mg. boric acid per kilogram body weight in rats does not alter normal estrus when given for twenty-one days.

Effect of Low Chloride Diets on Toxicity of Boric Acid.—The interesting suggestion made by Peyton and Green[3] that displacement of chloride might play a role in the toxicity of boric acid was investigated as follows:

Three groups of eight adult rats (from 170 to 220 Gm.) were formed. The rats in Group I were kept on Purina dog chow, those in Group II on a purified diet containing Wesson salt mixture, and those in Group III on a similar purified diet in which the chlorides of the Wesson salt mixture were replaced by sodium and potassium carbonates. All the rats were placed on the diets for one week prior to daily intraperitoneal injections of 4 c.c. of 4 per cent boric acid solution.

In the case of the rats in Group I, no deaths occurred during the three weeks of the experiment and no consistent changes in weights of the animals were noted.

The animals in Group II all soon became listless and died in from five to seven days. In Group III death of the animals occurred in from nine to sixteen days. Thus absence of chlorides did not increase susceptibility of rats to the toxic effects of boric acid. Blood chloride determinations were made on representative animals in Groups II and III. No lowering of blood chlorides was noted during the period of the experiment.

The excellent tolerance to boric acid of adult rats maintained on a normal stock diet was not explained but is conceivably due to the greater nutritive value of this type of ration over the purified diet. Whether the difference is a qualitative or quantitative one was not determined.

Preservative Properties of Boric Acid for Injectable Solutions.—The bacteriostatic effectiveness of boric acid was studied at levels of 0.15, 0.3, 0.5 and 1.5 per cent in solutions containing various B complex vitamins. Such solutions were shown to support growth readily in the absence of a preservative. Standard inocula (about 5,000 per cubic centimeter) of *Staphylococcus aureus*, *Escherichia coli*, and mixed mold spores were made in all cases. The samples were kept at room temperature. Colony counts were made from standard platings at regular intervals.

Levels of 0.3 and 0.5 per cent boric acid were effective in causing disappearance of inocula from B complex injectable solutions made to pH 4.5 and 5. Disappearance of bacteria was complete in two days; of mold, in twenty-eight days.

In the case of a solution containing 1.5 per cent boric acid and 0.1 per cent riboflavin buffered at pH 6.5, complete disappearance of viable bacteria required six days; of mold spores, thirty-five days. A comparison was made in this experiment against a riboflavin solution containing 0.5 per cent chlorobutanol as preservative. Although the chlorobutanol caused rapid disappearance of both bacterial strains, presence of mold spores was detected to the thirteenth day. A similar solution containing 0.15 per cent boric acid showed gradual but complete loss of bacterial inocula and considerable diminution of mold spores.

DISCUSSION

On the basis of its known toxicity, boric acid compares favorably with such commonly used preservatives for injectable solutions as phenol, the cresols, chlorobutanol, and benzyl alcohol. Furthermore, boric acid possesses the following advantages: low cost, high purity of U.S.P. crystals, lack of color or odor, chemical stability and inertness, and ease of solution. On the physiologic side it has the important advantages of being nonirritating to tissues and of being excreted rapidly in unchanged form.

The fact that boron is a normal food constituent and is an essential growth element for many plant species argues against its cumulative toxicity at very low levels of intake. The maximum tolerance level named by Wiley[1] of 0.5 Gm. per day for human beings is only six times the level reported by Kent and McCance[8] to represent a normal daily turnover from food sources. It seems likely that boric acid exerts toxic effects only when the renal threshold is exceeded and accumulation occurs in the tissues. In all reports of acute toxicity in human beings, relatively massive doses of boric acid have been involved and there is a notable lack of reported cases of chronic toxicity. Thus the chronic cumulative type of toxicity caused by continued ingestion of minute amounts of compounds of lead, mercury, selenium, and fluorine, for example, is apparently not shared by compounds of boron, even though boric has a cumulative action in larger doses, that is, 0.5 Gm. daily.

The easy availability and very widespread use of boric acid by both members of the medical profession and laymen has led to its occasional fatal misuse. Extreme rapidity of assimilation through all membranes of the body, a fact which in many cases appears to have escaped attention, has also led to its misuse.

SUMMARY

Boric acid at from 0.5 to 1.5 per cent in injectable solutions showed no toxic effects in rats and dogs when injected over long periods. Levels averaging about 400 mg. per kilogram body weight per day for thirty days did not affect the blood picture or deter the growth of rats. Levels of from 60 to 70 mg. per kilogram appeared to have no effect on the estrus cycle.

Varying the level of chlorides in the diet did not appear to influence the toxicity of injected boric acid; however, it appears likely that other nutritional factors play a part in tolerance for this drug.

The bacteriostatic effect of boric acid, from 0.5 to 1.5 per cent, against selected bacteria and molds appeared satisfactory for preservation of certain injectable solutions.

The bacteriologic work and the long-term nutritional experiments were carried out by Dr. H. W. Cromwell and F. Peirce Dann, to whom we are indebted.

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RELATIONSHIPS OF CHEMICAL CONSTITUTION TO THE ANTIBACTERIAL EFFECTS OF DERIVATIVES OF 9-AMINOACRIDINE

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THE antibacterial effects of various acridine compounds, in vitro, have been presented in two previous reports from these laboratories.[1, 2] Of significant interest were the comparatively low concentrations of 9-aminoacridine required to exhibit bacteriostatic and bactericidal properties against a variety of organisms commonly associated with wound infections. A 3-chloro-7-methoxy derivative of 9-aminoacridine proved to have even greater antibacterial effects than the parent compound, particularly against members of the anaerobic clostridium group. It seemed desirable, therefore, to study additional derivatives of 9-aminoacridine and especially those containing the 3-chloro-7-methoxy groups. The compounds tested, together with their molecular weights, are as follows:

1. 3-methyl-7-methoxy-9-(1-methyl-4-diethylamino)butylaminoacridine dihydrochloride (molecular weight, 452.5)
2. 3-chloro-7-methoxy-9-(2-phenyl-4-dimethylaminobutylamino)acridine dihydrochloride (molecular weight, 506.5)
3. 3-chloro-7-methoxy-9-(2-phenyl-4-diethylaminobutylamino)acridine dihydrochloride (molecular weight, 534.5)
4. 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino)butylaminoacridine tartrate (molecular weight, 549)*
5. 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino)butylaminoacridine formate (molecular weight, 491)*
6. 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino)butylaminoacridine citrate (molecular weight, 591)*
7. 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino)butylaminoacridine acetylsalicylate (molecular weight, 759)*
8. 3-chloro-7-methoxy-9-[(1-methyl-4-phenylpiperidyl-4-)methyl]amino acridine dihydrochloride (molecular weight, 536.5)
9. 3-chloro-7-methoxy-9-(4-dimethylamino)phenylaminoacridine dihydrochloride (molecular weight, 451)
10. 3-chloro-7-methoxy-9-[(1-benzyl-4-phenylpiperidyl-4)methyl]amino acridine dihydrochloride (molecular weight, 594.5)
11. 3-chloro-7-methoxy-9-(6′-methoxy-8′-quinolylamino)acridine dihydrochloride (molecular weight, 489)
12. 3-chloro-7-methoxy-9-(3-pyridylamino)acridine dihydrochloride (molecular weight, 409)

METHODS

The methods which were used in the two previous reports were followed in this investigation. This consisted essentially of preparing a series of dilutions

*Salts of Quinacrine, U. S. P.

From the Research Laboratories, Winthrop Chemical Co., Inc.
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TABLE I

HIGHEST DILUTION OF COMPOUND SHOWING ANTIBACTERIAL ACTIVITY

COMPOUND	PNEUMOCOCCUS						STREPTOCOCCUS					
	TYPE I		TYPE II		TYPE III		HEMOLYTIC		VIRIDANS		AGALACTIAE	
	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc
1	256,000	256,000	512,000	512,000	256,000	256,000	256,000	32,000	64,000	8,000	64,000	16,000
2	256,000	256,000	512,000	512,000	256,000	256,000	128,000	64,000	64,000	4,000	64,000	32,000
3	256,000	128,000	1,024,000	256,000	128,000	16,000	128,000	64,000	128,000	8,000	256,000	128,000
4	256,000	128,000	512,000	256,000	128,000	128,000	128,000	64,000	32,000	16,000	32,000	32,000
5	128,000	128,000	256,000	128,000	128,000	128,000	128,000	64,000	32,000	16,000	64,000	32,000
6	128,000	128,000	256,000	128,000	128,000	128,000	128,000	64,000	32,000	16,000	32,000	32,000
7	128,000	32,000	256,000	128,000	128,000	128,000	128,000	16,000	16,000	8,000	32,000	16,000
8	128,000	64,000	256,000	128,000	128,000	64,000	256,000	32,000	64,000	32,000	32,000	<1,000
9	1,000	<1,000	<1,000	<1,000	1,000	<1,000	1,000	<1,000	1,000	<1,000	1,000	<1,000
10	<1,000	<1,000	1,000	<1,000	1,000	<1,000	1,000	<1,000	<1,000	<1,000	<1,000	<1,000
11	1,000	<1,000	2,000	<1,000	1,000	<1,000	2,000	<1,000	<1,000	<1,000	<1,000	<1,000
12	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000

Bs = Bacteriostatic.
Bc = Bactericidal.
<1,000 = Concentrations greater than 1:1,000 not tested.

TABLE II

HIGHEST DILUTION OF COMPOUND SHOWING ANTIBACTERIAL ACTIVITY

COMPOUND	STAPHYLOCOCCUS AUREUS		BACILLUS PYOCYANEUS		CLOSTRIDIUM							
					WELCHII		TETANI		HISTOLYTICUS		OEDEMATIENS	
	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc
1	2,000	<1,000	<1,000	<1,000	64,000	64,000	128,000	32,000	128,000	1,000	128,000	4,000
2	16,000	2,000	<1,000	<1,000	16,000	16,000	16,000	8,000	32,000	1,000	32,000	4,000
3	16,000	2,000	<1,000	<1,000	32,000	32,000	32,000	8,000	32,000	1,000	32,000	4,000
4	4,000	<1,000	<1,000	<1,000	8,000	8,000	8,000	2,000	16,000	<1,000	16,000	1,000
5	4,000	1,000	<1,000	<1,000	32,000	32,000	32,000	8,000	32,000	1,000	32,000	2,000
6	4,000	1,000	<1,000	<1,000	16,000	8,000	8,000	4,000	8,000	2,000	8,000	1,000
7	4,000	<1,000	<1,000	<1,000	8,000	8,000	16,000	2,000	8,000	4,000	8,000	<1,000
8	8,000	1,000	<1,000	<1,000	16,000	16,000	16,000	8,000	16,000	1,000	16,000	4,000
9	<1,000	<1,000	<1,000	<1,000	16,000	4,000	32,000	32,000	32,000	32,000	16,000	4,000
10	<1,000	<1,000	<1,000	<1,000	1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000
11	1,000	<1,000	1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000
12	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000

Bs = Bacteriostatic.
Bc = Bactericidal.
<1,000 = Concentrations greater than 1:1,000 not tested.

of each compound in an appropriate broth medium. After autoclaving the drug-broth solution, each dilution series together with a drug-free control, was inoculated with a loopful of a twenty-four-hour broth culture of one of the test organisms. The test organisms included types I, II, and III pneumococci, beta hemolytic streptococcus No. C-203, *Streptococcus viridans*, *Streptococcus agalactiae*, *Staphylococcus aureus* No. 209, *Bacillus pyocyaneus*, *Clostridium welchii*, *Clostridium tetani*, *Clostridium histolyticus*, and *Clostridium oedematiens*.

The inoculated tubes were incubated at 37° C. and examined for visible growth after twenty-four, forty-eight, and seventy-two hours. Failure of growth to appear in twenty-four hours was considered to be evidence of bacteriostasis. Tubes which failed to show growth after seventy-two hours were tested for the presence of living organisms by transferring three loopfuls to fresh medium lacking the drug. Lack of growth in the subculture tube was assumed to be evidence of a bactericidal effect on the part of the drug in the original drug-broth mixture. The results of these studies are given in Tables I and II.

RESULTS AND DISCUSSION

From data presented in Tables I and II, we note that compounds 1 through 8 are active against most of the bacteria tested. Compounds 9 through 12, however, are essentially devoid of any antibacterial activity. The acetylsalicylate, citrate, tartrate, and formate salts of quinacrine (compounds 4 through 7) displayed an antibacterial activity comparable to quinacrine itself. From the results obtained with compounds 9, 11, and 12, it would appear that substitution in the 9– position with an aromatic amine tends to decrease the activity of the compound markedly. By comparing compounds 8 and 10, we note that antibacterial effects are markedly increased by replacing the N-benzyl group (compound 10) with a methyl group (compound 8).

CONCLUSION

The activity of 3-chloro-7-methoxy-9-aminoacridine on bacteria is not increased by replacement of the amino group by certain N-containing alkyl or aryl amines. This conclusion is based upon in vitro studies of twelve such derivatives tested against various organisms which may be associated with wound infections.

The authors wish to express appreciation to Dr. C. E. Kwartler and Mr. P. Lucas for the preparation of some of the compounds. The methods of preparing the compounds will be presented elsewhere. Acknowledgment is also given to Virginia L. Wilson for technical assistance in the bacteriologic study.

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LABORATORY METHODS

A FRACTIONAL SUPPLEMENTARY TEST FOR DISTINGUISHING POSITIVE KAHN REACTIONS IN SYPHILIS AND MALARIA

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FALSE reactions in serologic tests for syphilis resulting from malaria have been recognized by many workers. With troops returning to civilian life from malarious districts, the problem becomes more acute not only because of the stigma attached to the "positive" serologic report, but also for diagnostic reasons. The expected number of false positive reactions obtained with the standard Kahn test on malarial individuals is as high as 47.5 per cent at some stages of the disease.[1]

The fact that several diseases are known to be responsible for false reactions has brought about investigation of the so-called confirmatory tests, the purpose of which is to verify the fact that syphilis alone is responsible for the positive reaction. Kahn[2-4] has published details of three such techniques, the basis of the tests being, respectively, (1) differential temperature, (2) differential electrolyte concentration, and (3) salt dispersibility. Chargin and Rein[5] commented favorably on the first method as a result of their studies. Discrepancies were observed in some blood specimens subjected to repeated "verification tests." De Groat,[6] after submitting malarial bloods to Kahn's laboratory for the differential temperature verification test, believes the test valuable in this disease. No discussions of the other two methods have been found to date. Rytz[7] also has published a differential method which removes from serum all but traces of protein by the use of a weak copper sulfate solution. Upon the deproteinized substance is performed the Rytz flocculation test. No data concerning malaria were presented.

I[1] observed that with serums of malarial individuals a constant pattern of reactivity is found when several standard serologic tests are performed. The fact that the Eagle flocculation test and the Hinton test are usually negative when the Kahn test is either doubtful or positive suggested that there existed an actual difference in the character of the immunologic substances causing flocculation in syphilis as contrasted with those in malaria. Since the standard Kahn test is used so extensively, and also because it exhibits a high incidence of false positives in malaria, it was made the basis of a new confirmatory procedure. The serum rather than the other factors was modified.

The possibility was considered that the flocculating substance induced by the malaria and the syphilitic "reagin" might be associated with different serum protein fractions. Therefore precipitation of malarial and syphilitic sera, both

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of which gave positive Kahn reactions, was carried out employing various quantities of saturated ammonium sulfate. The several fractions thus obtained were each tested by the Kahn standard test. It was found that syphilitic reagin was attached to the first fraction brought down, whereas the malarial antibody was not so fixed. In other fractions, however, both antibodies were present in varying quantity. Upon this point then is based the following supplementary test which differentiates between a true syphilitic blood and a blood with a positive reaction induced by malaria. This procedure is advantageous in that it can easily be performed in laboratories equipped to do the standard Kahn test.

METHOD

A saturated solution of ammonium sulfate as used in the Kahn test on spinal fluids is required. No other special apparatus or solutions are essential.

The standard Kahn test is first performed on all sera to determine the degree of positivity. Only specimens free of hemolysis should be employed.

To 1 c.c. of inactivated serum in a Kahn tube at room temperature is added exactly 0.35 c.c. saturated ammonium sulfate solution. It is necessary to dry the outside of the pipette used to measure the saturated solution prior to its addition in order to insure that no excess is added. A 1 c.c. serologic pipette graduated in hundreths is used for this purpose. No crystalline ammonium sulfate should be pipetted over. The tube is tapped to mix the substances thoroughly and allowed to stand for, and not more than, two minutes, after which the tube is centrifuged at from 1,000 to 2,000 r.p.m. for three minutes (keeping switch in the "on" position for three minutes). Failure to comply strictly with the quantity and the time stated will result in the precipitation of serum fractions which will yield false positive results.

Following the centrifugation the tube is inverted to discard the supernatant fluid and while still in the inverted position is allowed to drain on filter paper for one minute. The precipitate will adhere to the bottom of the tube.

Physiologic saline (0.9 per cent) is then added to the precipitate, the quantity depending on the original Kahn test reading as follows:

If the reading was doubtful (± or 1+), add 0.7 c.c. saline.

If the reading was positive (2+ or 3+), add 0.9 c.c. saline.

If the reading was positive (4+), add 1.2 c.c. saline.

Negatives (see under Discussion) may be tested by assuming that the serum gave a "doubtful" result.

After several minutes, assisted by gentle tapping, the precipitate goes completely into solution. The fractionation is now completed and the testing of this fraction is carried out by performing a Kahn test in the standard manner. However, only the third tube of the three-tube test is employed, using 0.0125 c.c. antigen emulsion plus 0.15 c.c. of the solution containing the dissolved precipitate. The tube is shaken in a Kahn shaker for three minutes, 0.5 c.c. saline is added, and the tube reading is made immediately. Standard Kahn test readings of plus-minus, 1 plus, or 2 plus are given a "negative" report, whereas a 3 plus or 4 plus is given a "positive" report. A "positive" in this sense is interpreted to mean that the fraction carried syphilitic reagin; a "negative," that no reagin was originally present.

DISCUSSION

The results obtained in this laboratory are presented in Table I. Although the number of cases of malaria of necessity is omitted, the percentage values are based on all malarial patients giving false positive serologic results which occurred over several months. The diagnosis of malaria was confirmed in all instances by positive blood smears, and serologic "malarial patterns"[1] were obtained.

TABLE I

NUMBER OF CASES	DIAGNOSIS	STANDARD KAHN TEST POSITIVE AND DOUBTFUL (PER CENT)	FRACTIONAL SUPPLEMENTARY TEST POSITIVE (PER CENT)
*	Malaria	100	3.2
9	Syphilis (untreated)	100	100
50	Syphilis (treated)	100	90

*Number of cases deleted in accordance with censorship regulations.

Qualitative factors were more important than quantitative factors as far as the precipitation was concerned. This became evident when quantitative Kahn tests were done on all positives herein reported. Of the group of malarial sera, there were thirty-four with titers of 40 Kahn units or more which gave "negative" supplementary results while of the syphilitic sera there were nine "positives" with titers of less than 40 Kahn units, proving that isolation of the fraction definitely removes the malarial nonspecific-reacting substances.

It is of interest that in the treated patients with syphilis, 10 per cent of the sera were not positive in the supplementary test. This is evidence that the fraction usually carrying reagin may fail to do so in treated syphilis. The titer of reagin is again not determinative inasmuch as the sera of six untreated patients with syphilis with titers of 4 Kahn units were positive, whereas the sera of the five treated patients giving "negative" supplementary results had titers of 4 Kahn units or more. It is suggested, if this observation is confirmed and amplified, that it may be used as an index of complete cure in those patients who remain seroresistant after supposedly adequate treatment has been concluded. Serologic tests usually have been approved on the basis of specificity and sensitivity. It seems to be as important to evaluate tests on the basis of "persistency" also, which sets forth the concept that a continued positive in a completely treated and cured patient with syphilis is as false as any positive serologic reaction obtained from unrelated diseases. It should be noted that Rytz[7] reported of his differential method that sera of patients with syphilis treated for more than a year and a half gave a negative confirmatory reaction although routine serologic tests were positive.

As a finding of interest, mention should be made of two syphilitic bloods which gave negative results in the standard Kahn test and positive results in the Hinton tests. Upon removal of the fractions used in the supplementary test, these two serums gave "positive" results. Where the standard Kahn failed to demonstrate a positive to begin with, reagin was nevertheless demonstrable after the first fraction was removed from the remainder of the serum under the conditions detailed for the present test. The failure of the reagin to be evident in the original serum may be due to the same phenomenon of inhibition

described by Brown.[8] In the present instance the inhibiting substance or substances were presumably removed. This finding also is in need of further consideration.

Patients with diseases other than malaria that produce false serologic reactions were not available at this installation so that the efficacy of this fractional supplementary test in other conditions is not known. It is logical to assume that if the linkage of antibody is the same as in malaria, the same results can be expected. These determinations remain to be carried out.

It is obvious that the fractionation is crude in that total protein and albumin-globulin ratios may vary from individual to individual.

CONCLUSIONS

1. A supplementary test for syphilis is described based on the isolation and testing of a fraction of the serum which carries with it syphilitic reagin and only rarely the immunologic substance or substances induced by an infection with malaria.

2. The fractional supplementary test is practical, easily performed, although demanding of accuracy in technique, and can be utilized by any laboratory routinely doing the standard Kahn test.

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COMPARATIVE RESULTS OBTAINED WITH THE KAHN STANDARD AND A ONE-TUBE FLOCCULATION PROCEDURE IN SEROLOGIC TESTS FOR SYPHILIS

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FOR the past four years advantage has been taken of the availability of the blood specimens submitted in the annual U. S. Public Health Service Evaluation of Serodiagnostic Tests for Syphilis to determine the sensitivity and specificity of a single-tube diagnostic flocculation procedure as compared with the Kahn standard three-tube technique as carried out by Kahn and as performed in our own laboratory. Kahn standard antigen was used in all three procedures, but in the single-tube technique the proportion of patient's serum to antigen was 10:1, that is, 0.2 c.c. serum to 0.02 c.c. antigen. Otherwise, the technique of the author-serologist was followed meticulously, with the tests being read by artificial light and a mirror and the two readings, made at a fifteen-minute interval, averaged. In reporting the results of the one-tube test, readings averaging 2 plus or more were called positive; if the average was 1 plus, the test was called doubtful; and if the average was plus-minus (±) or negative, the report was negative.

The sensitivity and specificity ratings were worked out in Dr. Mahoney's office at the Venereal Disease Research Laboratory of the U. S. Public Health Service.

In Table I, *A*, it is indicated that 495 specimens from presumably normal, or at least nonsyphilitic individuals, were tested with no false positive or doubtful tests reported by Kahn or our own laboratory, using the Kahn standard test. One false positive and no false doubtful result was obtained with the one-tube method.

In Table I, *B*, are given the results in 156 cases of early syphilis. Of these, Kahn found 110 positive and we found 112 with the three-tube test. With the one-tube test, 111 were positive.

In Table I, *C*, are shown the findings in 371 cases of syphilis in the latent stage. Kahn detected 265 and we detected 246 with the standard test, whereas 262 were positive with the one-tube technique.

In Table I, *D*, are included 304 cases of late syphilis. Kahn found 258 and we found 239 positive with the Kahn standard procedure, while with the one-tube test we detected 250.

In Table I, *E*, are shown the results on sera from 43 cases of congenital syphilis. In this group Kahn detected 36 and we detected 30 with the Kahn standard test. With the one-tube method we picked up 34.

In Table I, *F*, are summarized the findings in 874 sera from syphilitic patients. Of these, Kahn found 669 and we reported 627 positive with the three-

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tube technique, while with the one-tube test we found 657 positives. In *F* it is also indicated that, in our hands at least, significantly fewer doubtful tests were reported with the one-tube than with the three-tube test.

TABLE I

RESULTS OBTAINED IN THE LABORATORY OF DR. R. L. KAHN AND THE ALABAMA STATE DEPARTMENT OF HEALTH USING THE KAHN STANDARD FLOCCULATION TEST AND A ONE-TUBE TECHNIQUE (SPECIMENS SUBMITTED IN THE U. S. PUBLIC HEALTH SERVICE EVALUATION OF SERODIAGNOSTIC TESTS FOR SYPHILIS, 1941, 1942, 1943, 1944)

SURVEY	SPECIMENS	KAHN STANDARD			ALA. KAHN STANDARD			ALA. ONE-TUBE		
		POS.	DOUBT.	NEG.	POS.	DOUBT.	NEG.	POS.	DOUBT.	NEG.
				Nonsyphilitic						
A	495			495			495	1		494
				Early Syphilis						
B	156	110	9	37	112	5	39	111	7	38
				Latent Syphilis						
C	371	265	16	90	246	22	103	262	12	97
				Late Syphilis						
D	304	258	12	34	239	19	46	250	13	41
				Congenital Syphilis						
E	43	36	1	6	30	4	9	34		9
				Syphilitic (Summary)						
F	874	669	38	167	627	50	197	659	32	185

In Table II is given the comparative standing as to sensitivity and specificity of the three-tube and one-tube tests for four years in the U. S. Public Health Service Annual Evaluation Study.

TABLE II

RESULTS OBTAINED IN THE LABORATORY OF DR. R. L. KAHN AND THE ALABAMA STATE DEPARTMENT OF HEALTH USING THE KAHN STANDARD FLOCCULATION TEST AND A ONE-TUBE TECHNIQUE (SPECIMENS SUBMITTED IN THE U. S. PUBLIC HEALTH SERVICE EVALUATION OF SERODIAGNOSIS TESTS FOR SYPHILIS, 1941, 1942, 1943, 1944)

YEAR		SENSITIVITY	SPECIFICITY
1941	Dr. Kahn standard	79.2	100.0
	Alabama standard	67.0	100.0
	Alabama one-tube	72.2	100.0
1942	Kahn standard	80.7	100.0
	Alabama standard	75.5	100.0
	Alabama one-tube	78.3	100.0
1943	Kahn standard	71.8	100.0
	Alabama standard	70.9	100.0
	Alabama one-tube	71.7	100.0
1944	Kahn standard	82.1	100.0
	Alabama standard	84.7	100.0
	Alabama one-tube	83.6	99.4

CONCLUSION

A one-tube test for syphilis, using patient's serum and Kahn standard antigen in a 10:1 ratio, is essentially as sensitive and as specific as the three-tube Kahn standard technique, has the advantage of giving fewer doubtful reactions, and is simpler and easier to perform.

AN IMPROVED METHOD FOR DETERMINING THE COAGULASE ACTIVITY OF STAPHYLOCOCCI BY MEANS OF THE PLASMA AGAR PLATE

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THE observation of Loeb[1] that certain strains of staphylococci were able to coagulate goose plasma was followed a few years later by the demonstration of the relationship of that property of the staphylococci to their pathogenicity.[2] Through the studies of numerous other workers,[3-9] coagulase production has been accepted as a means of ascertaining the possible importance in disease production of staphylococci isolated from human sources. In many clinical laboratories this test has become a routine procedure, either replacing or supplementing the determination of hemolytic activity and mannite fermentation.

The usual procedure for determining coagulase production is a test tube method in which the staphylococci are inoculated into the diluted plasma. The tubes are incubated at 37° C. and read at the end of three and eighteen hours. While this procedure is not laborious, it does require that sterile plasma be kept on hand and that the dilutions be prepared each time a determination is made. Furthermore, the organism must be isolated in pure culture, which entails a delay in reporting results.

In order to simplify the procedure used for coagulase determinations, it occurred to us that coagulase formation might possibly be determined in solid media to which plasma had been added. This would make possible the storage of plasma agar plates to be used as needed and also make possible a more rapid determination by the streaking of such plates directly with those specimens in which staphylococci had been observed in the direct smear preparations.

In the preliminary study, sterile plasma was added to equal amounts of previously sterilized and cooled veal infusion 4 per cent agar. This was well mixed by rotation and 20 c.c. amounts poured into sterile Petri dishes and allowed to solidify. The plates were inoculated heavily in scattered spots with twenty-four-hour cultures of staphylococci which had previously been tested for coagulase production by the test tube method. After overnight incubation at 37° C., there appeared around each colony known to be coagulase positive a gray halo of variable diameter. No zone appeared around the noncoagulase-producing staphylococcus strains. It was further noted that staphylococci growing on plates prepared in a similar manner, but using serum obtained from clotted blood instead of plasma, did not produce this halo effect but only a faint clear zone around certain of the strains. This eliminated the possibility that some substance other than coagulase was giving this halo reaction.

The gray halo in the plasma agar plates first appeared within three or four hours, at which time the majority of cultures could be read. In a few cases the

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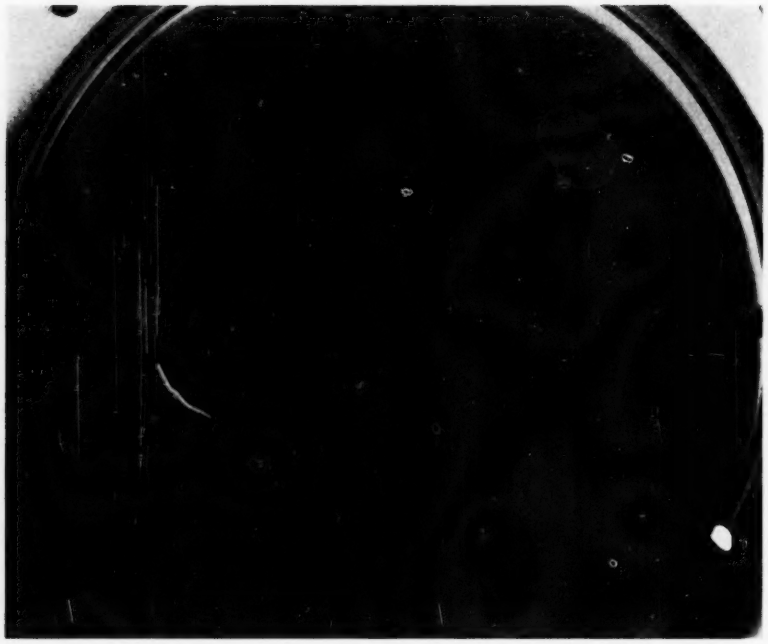

Fig. 1.—Plasma agar inoculated with coagulase-positive and coagulase-negative staphylococci. The production of a gray halo about the coagulase-positive strains differentiates them from the noncoagulase-producing staphylococci; eighteen-hour incubation at 37° C.

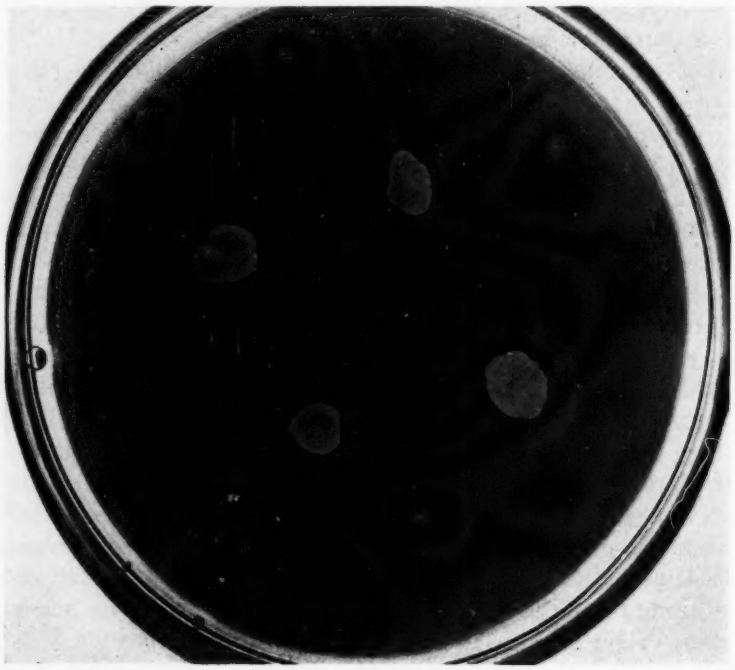

Fig. 2.—Plasma agar inoculated with staphylococci to show a second type of reaction that may occur with certain coagulase-positive strains. Note the area of clearing that is produced within the gray halo as contrasted with the reactions obtained in Fig. 1; eighteen-hour incubation at 37° C.

halo was not visible until the second reading at the end of eighteen hours. After overnight incubation, a zone of clearing was noted around many of those strains which were hemolytic on blood agar plates. Since it did not appear uniformly around all colonies which were known to be hemolytic, it could not be used as a criterion of hemolytic activity. However, nonhemolytic strains did not give this reaction. This clear zone did not interfere with the reading of the zone of coagulase formation but seemed to have pushed it outward so that the final appearance was a clear zone adjacent to the colony, about the periphery of which was a gray zone of variable diameter indicating coagulase activity. The various types of staphylococcus activity on plasma agar plates are shown in Figs. 1 and 2.

Prepared plates were stored for as long as thirty days and the reaction still occurred when these plates were inoculated with coagulase-positive staphylococci, thus indicating that deterioration in storage is not a great problem. Plates not over two weeks old, however, give the best results.

Various concentrations of plasma and types of media were used before obtaining one that we considered most suitable for routine use.

MATERIALS AND METHODS

Plasma Agar.—Veal infusion 2 per cent agar, containing 0.5 per cent peptone and 0.5 per cent sodium chloride, is prepared in the usual manner. This agar is sterilized and cooled to 43° C. As a fairly clear infusion agar is essential for easy reading of the reaction, the melted agar is poured into a sterile flask, leaving behind the sediment that may have accumulated. To this is added 20 per cent human plasma. We have made use of the plasma discarded by the blood bank because of positive serologic reactions. The agar is then poured into sterile Petri dishes in from 12 c.c. to 15 c.c. amounts and allowed to solidify. Prepared plates are stored in the icebox until ready for use.

Numerous strains of staphylococci can be tested for coagulase production on a single plate. Our usual procedure has been to divide the plate into sections and heavily inoculate an area approximately 4 mm. in diameter in each section with a staphylococcus growth obtained from solid media. The streak method of inoculation is also satisfactory, although the gray zone may be smaller due to the smaller size of the colonies. Incubation is carried out at 37° C. Reactions are recorded at the end of three hours and again after overnight incubation.

Where it is desirable to obtain an early report on the coagulase activity of staphylococci seen in the initial smears of infectious material received in the laboratory, direct plating of this material can be made on plasma agar plates and readings made the following morning. The streaking of the plate should be carried out in such a manner as to obtain isolated colonies. An example of this method of procedure is shown in Fig. 3.

Tube Method.—A loopful of culture from a twenty-four-hour veal infusion agar slant was inoculated into 1 c.c. of a 1:3 dilution of plasma. The tubes were incubated at 37° C. and read at the end of three and eighteen hours.

Hemolytic Activity.—Hemolytic activity was determined by inoculating the staphylococci from a twenty-four-hour veal infusion agar slant onto veal infusion

5 per cent sheep blood agar plates. The plates were incubated at 37° C. for twenty-four hours before reading.

Mannite Fermentation.—This was determined by the inoculation of mannite broth from twenty-four-hour cultures of the staphylococci grown on a veal infusion agar slant. The tubes were incubated at 37° C. and initial readings were made at the end of twenty-four hours with a final reading at the end of one week.

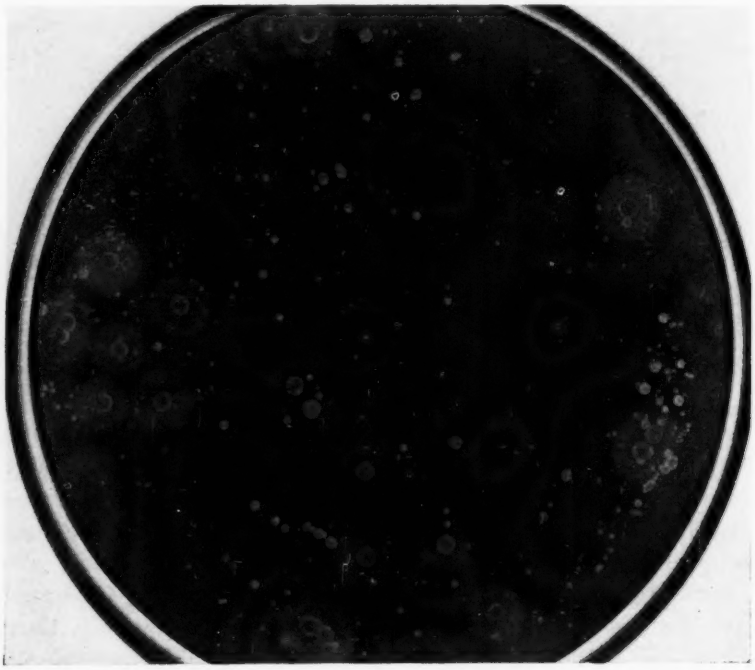

Fig. 3.—Plasma agar inoculated by streak method with sputum specimen containing coagulase-positive staphylococci; eighteen-hour incubation at 37° C.

RESULTS

Eighty-two strains of staphylococci, of which sixty-three were *Staphylococcus aureus,* seventeen *Staphylococcus albus,* and two *Staphylococcus citreus,* were used in this study. A comparative study of their ability to coagulate human plasma by the tube method and by the plate method described was made. The results are indicated in Table I together with such activities as mannite fermentation and hemolytic activity on blood agar plates.

Sixty-two of the *Staphylococcus aureus* strains were obtained from human sources and clinically were definitely related to the pathologic process from which they were isolated. One strain in this group was a stock strain used for testing the potency of penicillin preparations. All of these strains gave a positive coagulase reaction in eighteen hours by the tube and plate methods. It is to be noted, however, that in the three-hour reading of coagulase activity, only fifty-four of the strains gave a positive reaction by the tube method in this period of time. This was probably due to the fact that the plates are inoculated heavily in a small area, hence the beginning coagulase activity with certain strains is more quickly noted on the plate than in the tube, where the

TABLE I

RESULTS OBTAINED WITH EIGHTY-TWO STRAINS OF STAPHYLOCOCCI IN COMPARING THE VALUE OF THE TUBE AND PLATE COAGULASE TEST
(MANNITE FERMENTATION AND HEMOLYTIC ACTIVITY ALSO SHOWN)

SPECIES	NO. TESTED	MANNITE (NO. POS.)	HEMOLYTIC (NO. POS.)	TUBE COAG. (NO. POS.)		PLATE COAG. (NO. POS.)		SOURCE
				3 HR.	18 HR.	3 HR.	18 HR.	
Staph. aureus	63	62	59	54	63	59	63	Definite pathologic process
Staph. albus	17	4	3	1	0	0	0	Various sources not related to pathologic process
Staph. citreus	2	0	0	0	0	0	0	

organisms are dispersed throughout the diluted plasma. The ability of this group of organisms to ferment mannite and to hemolyze red blood cells coincided well with coagulase production, although a few discrepancies in this respect can be noted in Table I.

The seventeen strains of *Staphylococcus albus* were from various sources which could not be correlated with an infectious process. None of them gave a positive coagulase test. As will be noted in Table I, there was some irregularity in the correlation between the activity of this group in mannite and on blood agar plates, although as would be expected in coagulase-negative strains, the majority of them were inactive in mannite and did not produce hemolysis on blood agar plates.

The two strains of *Staphylococcus citreus* were isolated from contaminated plates and were negative in all tests.

SUMMARY

The ability of certain strains of staphylococci to produce coagulase is a recognized means of determining their possible pathogenicity. This property is determined by inoculation of the staphylococci into tubes of diluted plasma. The tubes are then incubated at 37° C. and read for plasma clot formation at intervals of time, usually from three to eighteen hours. This procedure has the disadvantages of requiring sterile plasma to be kept on hand and dilution tubes to be prepared, as well as necessitating the isolation of the organism before the test can be made.

In order to simplify this procedure and to obtain rapid determinations for clinical laboratory reporting, we have developed a modified test which gives readings comparable with those obtained by the "standard" method. By the use of veal infusion agar plates to which 20 per cent sterile plasma has been added, many of the disadvantages of the tube method of coagulase determination can be obviated. Coagulase-producing staphylococci inoculated onto such plates produce a gray halo effect about the inoculum within three to eighteen hours. Noncoagulase-producing staphylococci do not give a reaction. Numerous strains of staphylococci can be tested on a single plate. Plates of this medium can be prepared and stored for use as needed, eliminating the necessity of preparing dilution tubes each time a test is run.

For clinical laboratory purposes where it is desirable to obtain an early reading on the coagulase-producing power of a staphylococcus seen in smears of infectious material, this material can be streaked directly on plasma agar

plates. After overnight incubation of these plates the coagulase-producing activity of the organism can be determined by observing single isolated colonies. This eliminates the necessity of isolating the organism before the determination is made. Infectious material in which numerous other organisms besides staphylococci are present can be handled in a similar manner as long as isolated colonies of staphylococci are obtained, since we have not observed any interference by other organisms with this reaction.

As this media supports the growth of many of the pathogens such as streptococci, pneumococci, and meningococci, it may also be found useful as routine culture media for clinical laboratories.

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SIMPLE TECHNIQUE FOR ESTIMATION OF PENICILLIN IN BLOOD AND OTHER BODY FLUIDS*

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THE Oxford cup method for the assay of penicillin, while applicable to fluids containing fairly high concentrations of the drug, is unsatisfactory for clinical determinations in the blood where the concentration is usually less than 1 unit per cubic centimeter. The method of Rammelkamp[1] is more commonly used for estimations in the blood but is laborious. Fleming's method, as described to us by Herrell,[2] of the Mayo Clinic, is simpler, and it is this procedure which has been further simplified by us. The three methods are basically similar. Each consists of serial dilution of the unknown blood and addition of a constant inoculum of hemolytic streptococci. After incubation the tubes are observed for evidence of inhibition of growth of the bacteria and compared with controls, similarly prepared, but containing known amounts of penicillin. The penicillin content of blood from man, dog, and rabbit, containing variously from 0.01 to 4 units per cubic centimeter, was repeatedly compared by the three methods with satisfactory agreement.

METHOD (BLOOD)

Unknown.—Set up eight small test tubes (Wassermann) in a suitable rack. To each, except the first, add 0.2 c.c. of physiologic salt solution. To the first and second tubes add 0.2 c.c. of the unknown whole (citrated) blood. From the second tube transfer 0.2 c.c. to the third tube, then from the third tube transfer 0.2 c.c. to the fourth, and so on, to make serial dilutions. Discard 0.2 c.c. from the final tube.

To each of the eight tubes add 0.2 c.c. of a suspension of hemolytic streptococci† in broth prepared as follows: Transfer streptococci from a twenty-four hour culture in broth, by loop, to fresh broth. Use one loop of streptococci for each cubic centimeter of broth, and prepare enough for all the unknown and control blood samples to be assayed.

Shake each tube, hold almost horizontally, and from it, by capillarity, nearly fill a glass capillary tube about 6 cm. long. While still holding the capillary tube horizontally, imbed the end in a layer of modeling clay, filling a groove in a small board. When all the capillary tubes are in place, in a row, turn the board so that the tubes are upright.

Incubate for twenty-four hours at 37° C., and then inspect the tubes for presence of hemolysis and colonies of streptococci.

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†Richards' strain or other fast-growing strain.

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Control.—Set up a series of tubes, as for the unknown blood, but use defibrinated horse blood containing 1 unit of penicillin per cubic centimeter in place of the unknown blood sample.

Estimation.—The most dilute control capillary tube without hemolysis and bacterial colonies indicates the dilution of 1 unit of penicillin which will inhibit the inoculum of streptococci. The preceding tube indicates the dilution of 0.5 units which will inhibit the streptococci and so forth. By comparison of the end point in the control series and that in the unknown series, the concentration of penicillin in the latter is estimated. Thus, if the end point in the control series is in the sixth tube, as it usually is, the unknown contains 1 unit, if it, also, gives the end point in the sixth tube; 0.5, if in the fifth tube, and so forth.

COMMENT

Sterilized glassware is used, but the tubes and pipettes are not flamed, except initially. To simplify preparation, the test tubes (Wassermann) are placed, without plugging, in a large wire rack, which is then wrapped in paper and sterilized. A small tear in one corner allows removal of individual tubes as needed. Any capillary tubes may be used, but in our hands the Kimble brand, No. 34500, for melting point determinations, has been most satisfactory.

Although the concentration of red cells differs in each tube, this does not affect the accuracy of the test. In this procedure the centrifuging of blood specimens and the addition of known amounts of red cells are eliminated. The simplification of the procedure, therefore, depends upon these omissions and upon the use of capillary tubes. Rabbit blood occasionally gives results in which the end point is difficult to determine, presumably because of delay in sedimentation of the red cells. Because penicillin is stated to enter red blood corpuscles in minute amounts only, any considerable shift in hematocrit would affect the concentration of penicillin when determined in whole blood, as in our procedure. However, the error seldom is significant, especially when considered in the light of the large inherent error of the serial dilutions.

The concentration of penicillin in other body fluids may be determined similarly. Urine must be diluted before estimation to bring the usually high urinary penicillin levels into the range of blood levels. It is usually diluted with nine parts of defibrinated horse blood, but broth may be used (the end point is then determined only on the basis of colony growth). The final values must, of course, be corrected for the dilution factor.

The age of the streptococci (up to five days), their dilution between 10^{0} and 10^{-4}, and the exact period over which the test is allowed to run have practically no effect upon the results obtained.

CONCLUSION

A simplification of Fleming's method for the estimation of penicillin is described.

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COMPARISON OF METHODS ADAPTABLE TO PRODUCTION LINE EXAMINATION OF SPUTUM FOR TUBERCLE BACILLI

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FOR several years there has been a need for some method of digestion and concentration of tubercle bacilli in sputum as a routine procedure in the Division of Laboratories of the Tennessee Department of Public Health. Some of the factors considered desirable were: (1) a simple method of concentration adaptable to use on many specimens; (2) sufficient increase in positive findings to make the procedure worth while; (3) elimination of extraneous material which might interfere with the identification of a few organisms in the microscopic field.

Several methods were available which might prove satisfactory. Accordingly, a series of examinations was made to determine which of these methods should be employed routinely. Preliminary checks had been made by Brougher and one of us,[1] comparing the direct smear with the autoclave (heat coagulation) method and the trypsin digestion method. The autoclave method was found more efficient in these studies than the trypsin method. This fact, together with the safety and simplicity of the autoclave method, allowed its choice in preference to the trypsin digestion for further study.

Robinson and Stovall (1941)[2] compared NaOH digestion and concentration with a number of other methods listed as follows:

1. Alkalies, other than NaOH, as calcium hydroxide and ammonium hydroxide
2. Acids (6 per cent sulfuric)
3. Animal ferments (trypsin)
4. Organic solvents ("tergitol penetrant 08" in solution with NaOH)
5. Vegetable ferments (caroid)

These authors make the following statement in regard to these methods: "From the study made it appears that the most satisfactory digesters are normal sodium hydroxide and caroid (2 to 5 per cent caroid with 0.2 to 0.1 per cent sodium hydroxide)." The use of caroid (commercial preparation of the enzyme papain), as presented by Sullivan and Sears (1939),[3] was not tried in this comparison of methods of concentration. Robinson and Stovall state: "Caroid, while it does not digest pus actively, is more satisfactory than trypsin." They suggest equal quantities of 0.1 to 0.2 per cent sodium hydroxide solution and a 5 or 2 per cent caroid solution as being more effective than either alone. They state further that caroid smears usually have an even background and good distribution of well-stained organisms, although decolorization is occasionally difficult. The advantages of this method, as described by the authors, are as follows: (1) rapid digestion; (2) no neutralization of digestant necessary; (3)

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sediment adheres well to glass slides; (4) cultures and animal inoculations may be made from sediment after treatment to destroy other organisms.

After comparing the methods of digestion and concentration which had been studied they conclude: "The wide variation in results obtained through the use of various methods of digesting sputa and gastric specimens and precipitating the tubercle bacilli from the digests indicates that the newer methods of handling have little, if any, advantage over digestion with normal sodium hydroxide (2 per cent sodium hydroxide with gastric specimens or less mucoid sputa) and long centrifugation."

In view of these rather extended studies, it was evident that for our purposes more limited studies could be made. It was necessary to confine the work to a limited number of methods so that specimens could be divided and the digestion and concentration handled for simultaneous staining by each of the methods being compared. No diluted chemical flocculation[4] or volatile hydrocarbons were used in the studies presented to aid in collection of the organisms. The methods finally chosen for comparison were: (1) direct smear, (2) autoclave (heat coagulation) and concentration, (3) NaOH digestion and concentration,[5] and (4) clorox digestion and concentration.[6]

EXPERIMENTAL PROCEDURE

Routine specimens received in the Central Laboratory were used in this study. Direct smears were made routinely for diagnosis. By means of an applicator a sample of the most promising material was secured for smears. Concentration techniques were used for further study of the specimens. The direct smears were then compared with those prepared by concentration techniques.

Although all specimens were supposed to have been submitted in 5 per cent phenol, in many cases the disinfectant was discarded from the specimen vial before collection of the sputum. The presence or absence of phenol did not interfere in any way with the methods employed. Specimens amounting to at least 5 to 10 c.c. were selected for comparison.

Three series of tubes were prepared. Each specimen was divided by means of a 1 c.c. pipette into three equal parts. Only those specimens which could be divided satisfactorily were used. An occasional very thick mucoid specimen was included. In order to divide such specimens it was necessary to add a small amount of N/1 NaOH to digest the heavy mucus. There was no indication in these few specimens that the NaOH added for digestion interfered in any way with the efficiency of any of the methods. It no doubt aided digestion to some extent.

The sputa in the first series of tubes were used for autoclave (heat coagulation) and concentration. To these tubes an equal volume of 5 per cent phenol was added. The specimens were held in the autoclave at 15 pounds pressure for from twenty to thirty minutes. They were then stirred well before being centrifuged.

The sputa in the second series of tubes were used for NaOH digestion and concentration. To this series equal volumes of N/1 NaOH were added. Each specimen was stirred vigorously with an applicator to hasten digestion. The tubes were then placed in the incubator for at least thirty minutes. Digestion

for from one to three hours had no ill effects. Digestion was aided by shaking or stirring the specimens at least once during incubation. Following incubation, 1 to 2 drops of phenol red indicator were added to each specimen. Sufficient 3 per cent HCl was added to neutralize the specimens. Then, a few more drops were added until the reaction was definitely acid. In practically all cases a good flocculation was obtained.

The sputa in the third series of tubes were used for clorox digestion and concentration. To this series equal volumes of clorox were added. The specimens were stirred well with applicators, allowed to stand from ten to thirty minutes, and stirred well again before they were centrifuged.

Timing was arranged so that the three series of tubes were ready for the centrifuge at the same time. The specimens were centrifuged at 3,000 r.p.m. for thirty minutes.

Three sets of new, chemically clean slides were used. Smears were made from the centrifuged sediment after the supernatant was discarded. An applicator, prepared with a wisp of cotton, was used to make the smears. After air drying and fixation by heat, all slides were stained by the Ziehl-Neelsen method.

Slides were placed on a rack, carbolfuchsin added, and the preparations steamed for five minutes. After cooling for ten minutes, they were washed with tap water and decolorized with acid alcohol. They were then washed again and counterstained with Loeffler's methylene blue, diluted 1:4 with distilled water. Undiluted Loeffler's was found to give too deep staining for all organisms to show up well and the diluted counterstain gave sufficient background for contrast.

Specimens found negative by routine direct smear and positive by concentration method were rechecked by direct examination. Except where otherwise specified, observations include the recheck.

OBSERVATIONS

Two hundred and eleven specimens were examined. Of these, seventy-five were found to contain tubercle bacilli while 136 were negative. The seventy-five sputa showing tubercle bacilli were collected in fifty-one instances for purposes of diagnosis and in sixteen as a check on treatment; for the remaining eight, no reason for collection was stated.

TABLE I

BREAKDOWN COMPARISON OF SEVENTY-FIVE POSITIVE FINDINGS

NUMBER	DIRECT	CLOROX	NaOH	AUTOCLAVE
1	+	+	+	−
4	−	−	−	+
9	−	+	−	−
2	−	+	+	−
4	−	+	−	+
45	+	+	+	+
9	−	+	+	+
1	+	−	−	−

Table I shows that one specimen was found positive by only the direct method, four by only the autoclave, and nine by only the clorox method. The other sixty-two specimens were found positive by two or more methods.

The figures in Table II present the total specimens found positive by each method used and the number out of seventy-five positives which were missed by each method.

TABLE II

SUMMARY OF POSITIVES AND NEGATIVES. SPECIMENS BY EACH METHOD FOR SEVENTY-FIVE POSITIVE BY ONE OR MORE

METHOD	NUMBER POSITIVE	NUMBER MISSED
Direct without recheck	39	36
Direct rechecked	47	28
NaOH	57	18
Autoclave	62	13
Clorox	70	5

The forty-five specimens positive by all of the methods were used in compiling the figures in Tables III and IV. The organisms found represent the total in all the fields that it was necessary to examine by the method indicated in order to call these specimens positive. The greater number of fields listed in Table III for the direct microscopic method as compared with the others is due to the fact that in the direct method there was no concentration; therefore, it is often necessary to search more fields to find acid-fast organisms. Similarly, more fields had to be searched with the NaOH and autoclave methods than with the clorox method.

In Table III the number of organisms per 100 microscopic fields examined is an average determined by examination of the forty-five specimens found positive by all four methods.

TABLE III

AVERAGE NUMBER OF ACID-FAST ORGANISMS PER 100 FIELDS FOR FORTY-FIVE SPECIMENS FOUND POSITIVE BY ALL METHODS

METHOD USED	TOTAL FIELDS EXAMINED	TOTAL ORGANISMS FOUND IN FIELDS EXAMINED	AVERAGE NUMBER OF ORGANISMS PER 100 FIELDS
Direct	1,030	599	58
NaOH	316	1,266	401
Autoclave	244	1,045	428
Clorox	91	3,518	3.874

Listed in Table IV are the number of specimens, by methods, out of the seventy-five positive sputa for which the highest total average count of organisms per microscopic field was secured. Where the highest average number per field was the same for two methods, each one has been credited.

TABLE IV

NUMBER SPECIMENS GIVING HIGHEST AVERAGE COUNT PER FIELDS EXAMINED

METHOD	NUMBER OF SPECIMENS
Direct	3
Autoclave	15
NaOH	13
Clorox	59

Only one stain was positive by direct smear which was not found positive by other methods.

DISCUSSION

From these observations, it may be seen that the clorox concentration method gave seventy positives of the seventy-five found to be positive by one or more of the methods tried. The NaOH digestion and autoclave (heat coagulation) methods did not vary greatly in efficiency. By these methods, fifty-seven and sixty-two specimens, respectively, were found to be positive.

In regard to average number of organisms found per 100 microscopic fields, it is quite apparent that the clorox method proved considerably more effective than the other methods, the autoclave method being next in order.

The results indicate that the clorox method is definitely superior to the direct method with the difference being statistically significant. Through combined evidence from the proportion found positive and organisms found, the value of the method seems evident. When one considers specimens separately, in fifty-nine of seventy-five it is found that the clorox method yielded the highest count obtained per microscopic field. Only one specimen was found positive by direct smear which was missed by the other methods. The other four which were missed by the clorox method were positive only by the autoclave coagulation method.

The use of chemical flocculation or volatile hydrocarbon might have changed the picture somewhat. Since the Army Laboratories use Al $(OH)_3$ flocculation with the NaOH digestion method, it is felt that this modified procedure should be compared with the clorox digestion method for more complete information.

A study was therefore started with this in mind. It is proposed to make a further report on a series of specimens including 100 positives. This will allow for calculations of relative efficiency per 100 positive specimens.

Preparations made by the clorox digestion method had less visible extraneous material than did those made by the other methods used. When one observed a positive microscopic field in a clorox-digested preparation, the only formed elements seen were tubercle bacilli; there were no pus cells or other organisms. Further than this, it may be said that the clorox method is sufficiently simple so that it can be used readily in any laboratory which has a centrifuge. The method lends itself to use in a public health laboratory. Oliver and Reusser[6] report that the clorox method kills tubercle bacilli. This would make it a safe method although not satisfactory for cultures. No attempt has been made in this laboratory to use the clorox digestion and concentration method for culture work or animal inoculations.

SUMMARY

In this comparative study of four methods used to prepare sputa for the identification of tubercle bacilli, the clorox digestion method proved to be the most efficient. With it, the greatest percentage of positive specimens was obtained and there was a greater concentration of organisms per microscopic field. Because of this greater concentration, fewer fields had to be examined to find acid-fast bacilli. Furthermore, preparations so made have no visible extraneous material in the microscopic field to interfere with the search. The clorox digestion method lends itself readily to use in a public health laboratory where many specimens of sputum are received for microscopic examination.

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A RAPID STAINING TECHNIQUE FOR ACID-FAST ORGANISMS

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RAPID routine staining of acid-fast organisms with carbolfuchsin usually employs heat. Heat serves to produce more rapid penetration of the stain.

We have recently employed Tergitol No. 7* a detergent or wetting agent, as a substitute for heat and have found that it also accelerates the staining of acid-fast organisms. Staining occurs even more rapidly than with heat. Tergitol No. 7 was chosen because one of us had previously employed it successfully in histologic technique.[2]

A. TECHNIQUE FOR STAINING SMEARS

The following technique was applied to smears which included fresh sputum containing tubercle bacilli, concentrations of sputum prepared by the Petroff and phenol† methods, and pure cultures of tubercle bacilli.

Method.—The smears are fixed with heat and stained for one minute with modified Kinyoun's carbolfuchsin, the formula[1] for which is as follows:

Basic fuchsin	4 Gm.
Phenol crystals	8 Gm.
95 per cent ethyl alcohol	20 c.c.
Distilled water	100 c.c.
Tergitol No. 7	1 drop to every 30 to 40 c.c. of above mixture

Note.—This stain loses its desired rapid effect over a period of a few days. It is desirable to add the Tergitol No. 7 to the already mixed stain prior to use.

Next the smears are decolorized for one-half minute with acid alcohol, the following formula being used:

HCl	3 c.c.
95 per cent ethyl alcohol	97 c.c.

They are then counterstained for one minute with Loeffler's alkaline methylene blue, using the following formula:

Potassium hydroxide (10 per cent aq. sol.)	0.07 c.c.
Distilled water	70.0 c.c.
Stock solution methylene blue	30.0 c.c.

Note.—Methylene blue stock—1.48 Gm. to 100 c.c. of 95 per cent ethyl alcohol. Potassium hydroxide first added to water to make 1/10,000 dilution, then dye added.

Finally the smears are rinsed with water and dried.

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*Tergitol No. 7 obtained from the Carbide and Carbon Chemical Corporation, New York, N. Y.

†Phenol method for tubercle bacilli concentration in sputum: To the sample of sputum to be concentrated, an equal volume of 5 per cent phenol is added. This is mixed well and autoclaved at 15 pounds pressure for fifteen minutes. The specimen is cooled, the upper portion is decanted, and the remainder centrifuged at 1800 r.p.m. for from ten to fifteen minutes. The sediment is streaked on the slide, dried by air, fixed with heat, and stained.

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This staining method was found to give more consistent results than were obtained with the standard Ziehl-Neelsen method. The acid-fast organisms stained more intensely, were more easily discernible in the field, and the detail of the individual organism was more pronounced. This was especially noticeable with the beaded bacillary form of the tubercle bacillus. Moreover, in certain instances we have been able to demonstrate acid-fast bacilli with the method described in known tuberculous infections when similar preparations were negative where heat was employed instead of Tergitol No. 7. In the staining of smears, the substitution of a 1.5 per cent solution of fast green as a counterstain for Loeffler's alkaline methylene blue was found to be very satisfactory.

Several experiments were performed in an attempt to test the specificity of the method for acid-fast organisms. A hundred sputum smears known to contain no tubercle bacilli were stained by the new technique and examined for possible false staining reactions. No acid-fast organisms were found. The stain was then applied to smears made from pure cultures of a wide variety of the common bacterial genera, and again no false reactions were observed. Sputum smears collected from other laboratories and found to contain tubercle bacilli when stained by the standard methods were also positive in all instances when stained by the new technique.

B. TECHNIQUE FOR STAINING TISSUE SECTIONS

The use of the new modified stain was applied to sections of tissue known to be infected with *Mycobacterium tuberculosis*. The staining process was again accelerated and excellent differentiation of the organisms was obtained after five minutes' staining. One difficulty encountered, however, was the selection of a counterstain which would give morphologic detail and sharp contrast with the organisms. Loeffler's alkaline methylene blue gave poor results and little contrast. Heidenhain's hematoxylin differentiated the tissues adequately, but the similarity of color to the acid-fast stain made it difficult to distinguish the organisms. After testing a variety of stains, fast green, described in the following paragraphs, was selected as the most desirable counterstain. In preparations counterstained for two minutes with fast green, the organisms stood out so clearly against a green background that they could be identified easily under high dry magnification.

Method.—Tissues are fixed in formalin for twenty-four hours, embedded in paraffin by any of the standard techniques, sectioned, and mounted on slides. The slides are immersed in the following:

1. Xylol for three minutes
2. Xylol for two minutes
3. 95 per cent ethyl alcohol for two minutes
4. 80 per cent ethyl alcohol for two minutes
5. 70 per cent ethyl alcohol for two minutes
6. 50 per cent ethyl alcohol for two minutes
7. 30 per cent ethyl alcohol for two minutes
8. Distilled water for two minutes and
9. Modified Kinyoun's carbolfuchsin stain, prepared as previously described, for five minutes

They are then rinsed with distilled water, decolorized with acid alcohol until the tissues are light pink in color, and counterstained with fast green for two minutes. The fast green staining solution is prepared by adding 1.5 c.c. of a saturated alcoholic solution of fast green to 98.5 c.c. of distilled water. After the counterstaining, slides are again rinsed with water, immersed in 95 per cent ethyl alcohol for one minute, absolute ethyl alcohol for two minutes, xylol for two minutes, and in a second jar of xylol for five more minutes. Coverslips are applied with balsam or gum damar.

If the hematoxylin-carbolfuchsin method is used, the slides are prepared as through step 8 above. The tissue is then stained with Harris hematoxylin for ten minutes. This time may be decreased to from 1.5 to 3 minutes by adding 1 drop of Tergitol No. 7 to each 30 to 40 c.c. of the hematoxylin stain. The slides are next rinsed in tap water and destained with acid alcohol prepared by adding 1 c.c. of concentrated hydrochloric acid to 100 c.c. of 75 per cent ethyl alcohol. They are again rinsed in tap water and immersed in ammonia water (2 to 3 drops of ammonium hydroxide in 100 c.c. of distilled water) for five minutes. After a third rinsing in water, they are stained for five minutes with the modified Kinyoun's carbolfuchsin stain, rinsed in water, destained with acid alcohol (concentrated hydrochloric acid, 3 c.c.; 95 per cent ethyl alcohol, 97 c.c.) and rinsed a final time. They are then immersed in 95 per cent alcohol for one minute, absolute alcohol for two minutes, xylol for two minutes, and a second xylol jar for five minutes. Coverslips are applied with balsam or gum damar.

CONCLUSION

Rapid staining techniques for acid-fast organisms in smears and tissues are presented. The detergent Tergitol No. 7 was used to replace heat. The shorter time required for staining and the ease with which the procedure can be employed constitute improvements in technique which make the methods particularly applicable to routine use. Equal or better results are obtained with these procedures than with the older methods, and none of the required specificity for acid-fast organisms is lost.

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GERHARDT'S TEST FOR ACETO-ACETIC ACID IN URINE

A Simplified Method for Distinguishing Between True and False Positive Reactions

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IN ORDER to distinguish between a positive Gerhardt test due to aceto-acetic (diacetic) acid and one due to certain drugs, for example, acetylsalicylic acid (aspirin), sodium salicylate, phenacetin, phenazone (antipyrine), one of the following tests is usually performed:

1. A fresh portion of the urine is boiled and the test repeated. If the color was due to a drug, the second test will also be positive; if due to aceto-acetic acid, the second test will be negative. As pointed out by Harrison,[1] this method is reliable only if the urine is boiled vigorously in a large boiling tube or in an open vessel for several minutes.

2. The colored solution is boiled for a few minutes. If the color is due to aceto-acetic acid, it will disappear; if due to a drug, it remains.

As Todd and Sanford[2] point out, however, "it is not sufficient to boil the urine after the color has been brought out by the ferric chloride, as is sometimes advised, since the color caused by certain of the drugs will then disappear as well as that caused by diacetic acid."

The following modified technique, the outcome of experiments in this department, is recommended as a routine procedure.

Gerhardt's Test.—Add about 4 c.c. (1-inch column) of urine to 2 or 3 c.c. (½-inch column) of 10 per cent ferric chloride solution in a test tube. It should be noted that aceto-acetic acid and all the drugs mentioned, except phenacetin, give an immediate positive reaction. The dark brown color due to phenacetin develops slowly and only reaches its maximum intensity after two or three minutes.

If the test is positive, add 1 drop of concentrated nitric acid to 1 inch of urine in a test tube and boil for about one minute. Cool thoroughly under the tap and add ½ inch ferric chloride solution.

1. If the urine contains aceto-acetic acid, the second test will be negative.

2. If the urine contains a drug, the second test will be positive; when moderate or small amounts are present, the color may be slightly reduced in intensity.

3. If the urine contains both aceto-acetic acid and a drug, the result obtained will depend on the concentration of the drug. With moderate or small amounts the color will be much less intense in the second test. If large amounts of a drug are present, the color will still be so intense after boiling with nitric acid that any fading of the color due to the disappearance of aceto-acetic acid may not be perceptible.

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THE CONSTRUCTION, CALIBRATION, AND USE OF AN ALTERNATING CURRENT ELECTRODYNAMIC BRAKE BICYCLE ERGOMETER

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MANY types of ergometers have been employed for the purpose of investigating physiologic problems relating to the working efficiency of the human body. Faulty design, poor construction, and crude computations limited the practical usefulness of many of these devices. Methods of measuring the amount of work done by the arms included lifting weights, shoveling sand and coal, rowing, pumping water, and turning a crank, and various forms of ergometers were used. In problems involving the use of the legs, climbing of stairs, walking on a treadmill, wheeling a loaded wheelbarrow up an incline, and riding a bicycle have been employed extensively.

Recent attempts in this field of research have shown that the bicycle ergometer has many advantages over other methods of measuring work output. The first advantage is in the muscle groups involved in pedaling. The extensor muscles of the legs are the strongest and most highly developed muscles of the body and consequently make possible much higher rates of working, prolonged periods of working, and a greater total output. The second advantage lies in the fact that the head and thorax of the working subject are more or less stationary. This is a distinct advantage in collecting continuous data on respiratory reactions and in making observations relative to physiologic changes during the activity period. Other advantages arise from the inherent features of the machine which make possible controlled variations in the load, constancy in the rate of working, and accurate and automatic recording of data.

Bicycle ergometers which have been constructed by various investigators in the past have been of two types. A friction brake was used in the earliest models. Due to uneven braking action and uncontrollable loss of heat, the friction brake did not prove to be a satisfactory measuring device. The results are grossly inaccurate, and refinement of technique, which might partially overcome these deficiencies, becomes exorbitant in time and expense. Recent models of the bicycle ergometer have utilized the principle of the electrodynamic braking action of the electrical generator. This latter form of bicycle ergometer has been developed and perfected and now provides us with a reliable, flexible, and completely automatic method of measuring the work output and power of the leg muscles.

The earliest investigations in the use of an electric brake bicycle ergometer were made by Atwater and Benedict,[1] who simply pressed the drive wheel of a small dynamo to the rear wheel of a bicycle and measured the current generated. This approach did not prove to be reliable for there was considerable slip in the contact between the drive wheel and the rear wheel of the bicycle.

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This made for uncertainty in the determination of the amount of work done. Furthermore, it was not possible to vary the working load or measure the constancy of the rate of working. Benedict and Carpenter[2] and Benedict and Cady[3] employed an electric brake bicycle ergometer which consisted essentially of a bicycle, the rear wheel of which was replaced by a copper disk. Turning the pedals caused the copper disk to rotate between the pole faces of an electromagnet and provided a constant source of resistance. The resistance or load could be varied by changing the magnitude of the current flowing through the coils of the electromagnet. This ergometer proved to be fairly accurate and reliable when properly used but had the disadvantage of being difficult to calibrate and did not provide a convenient method of recording data on work output. Kelso and Hellebrandt[4] designed a recording electrodynamic brake bicycle ergometer which was completely automatic, employing a direct current generator as the brake. In their ergometer the armature of the generator was connected to the pedal sprocket of the bicycle and the pedaling motion rotated the armature. The field coils of the generator were independently excited by a 12-volt storage battery, producing a magnetic field in which the armature revolved. When the revolving armature was placed in an electric circuit, in this case a resistor and a recording voltmeter in parallel, a current flowed through it. This current set up a magnetic field which opposed the magnetic field of the field coils, thereby producing the braking action of the ergometer. By varying the flow of current in the field coils, the braking action could be controlled accurately. Through careful calibration of the instrument it was then possible to interpret the terminal voltage of the armature in terms of work rate.

The apparatus herein described is essentially the same as that used by the last-mentioned investigators but has the added feature of being operated by alternating current. The current for exciting the field coils is obtained by means of a rectifier unit built into the circuit, thereby providing a reliable and convenient source of direct current.

CONSTRUCTION AND CARE OF THE ERGOMETER

In appearance and principle, the ergometer described herein is similar to that designed and constructed by Kelso and Hellebrandt.[4] In addition to the substitution of a rectifier unit to replace the storage battery for providing the field current, changes were made in the type and size of the various parts to make the machine more suitable for the purpose in mind.

Details of Construction.—The ergometer is portable, reliable, completely automatic, and of sturdy construction. Fig. 1 shows the over-all arrangement and placement of the various parts. Fig. 2 is a schematic wiring diagram and shows the electrical circuit used.

The framework is constructed of heavy steel girders and piping supported by four-inch, rubber-tired, ball-bearing castors. This construction provides a framework which is strong and rigid but still of high mobility, an important consideration since the ergometer is used in various parts of the laboratory. The seat, handlebars, frame, pedals, sprocket, and chain are parts of a high-grade bicycle. They are attached to the framework so as to simulate an ordinary bicycle. The seat and handlebars are movable to permit adjustments for body build.

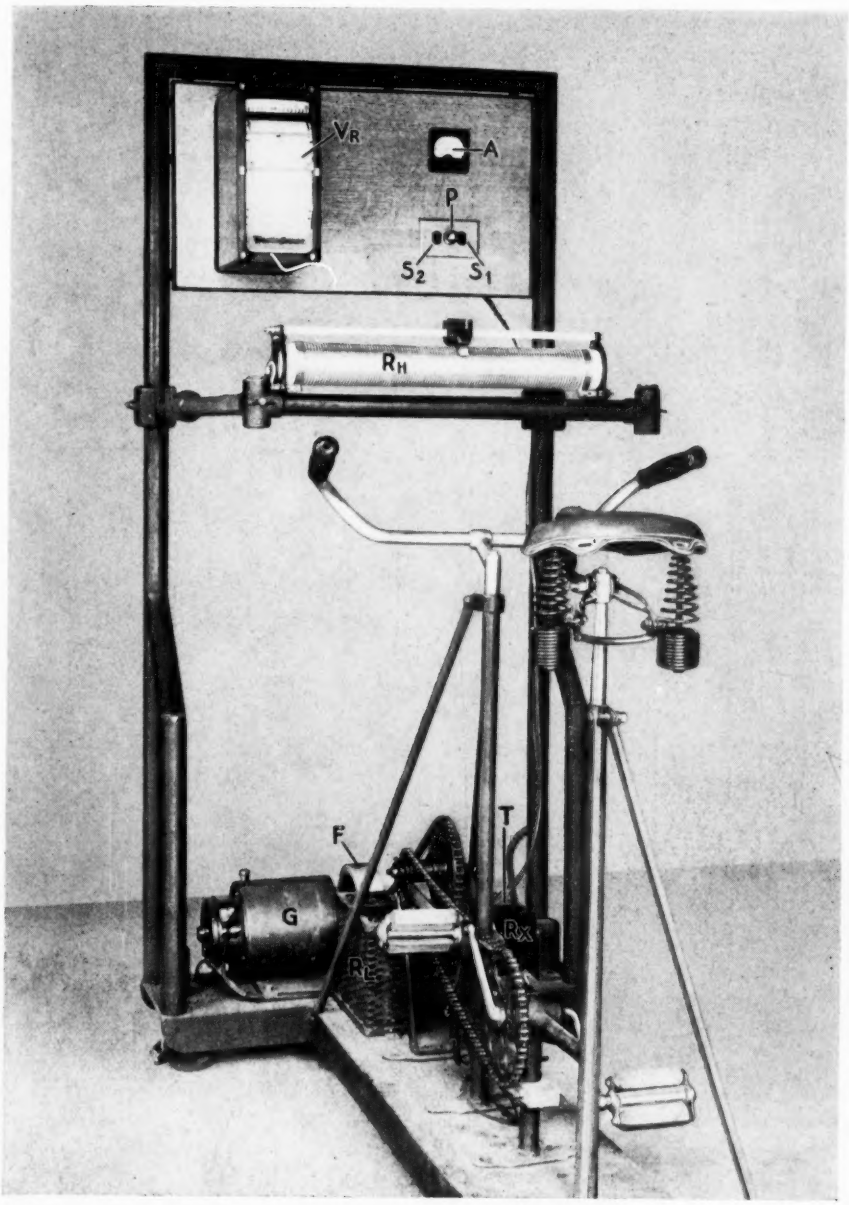

Fig. 1.—Photograph of the bicycle ergometer showing the placement of the main parts. V_R, Recording voltmeter; A, ammeter; P, pilot light; S_1 and S_2, switches; R_h, field rheostat; G, generator; F, flywheel; T, step-down transformer; R_1, load resistor; R_x, rectifier.

The pedal sprocket of the bicycle is connected to the drive shaft of a 12-volt direct current Dodge automobile starter-generator (G, Figs. 1 and 2) by means of a double sprocket and chain arrangement which gives a 1:12 ratio between the pedals and the generator armature. One revolution of the pedals, by this arrangement, results in twelve revolutions of the armature. An 8-inch flywheel (F, Fig. 1), weighing 15 pounds, is fastened to the armature drive shaft to provide rotary inertia for smooth pedaling. The series winding of the generator

is disconnected and is not used. The shunt winding is wired into the circuit so that it is independently excited to provide the magnetic field for the braking action of the generator.

The direct current for exciting the shunt winding, hereafter called the field coils (F, Fig. 2) of the generator, is obtained from a 110-volt alternating current source by means of a 32-volt step-down transformer and a Mallory magnesium-copper sulfide rectifying element (R_x, Figs. 1 and 2) in a single phase, full-wave bridge circuit. The rectifier is a type S36B9 with a maximum alternating current operating voltage of 28.7 and an inductive load of 12.8 volts (direct current) and 10.6 amperes (direct current). A 15-ampere direct current ammeter (A, Figs. 1 and 2) is placed in this circuit to measure the current flowing through the field coils. A 7.7-ohm, 12-ampere rheostat (R_h, Figs. 1 and 2) is included in the circuit to make adjustments of the field current possible.

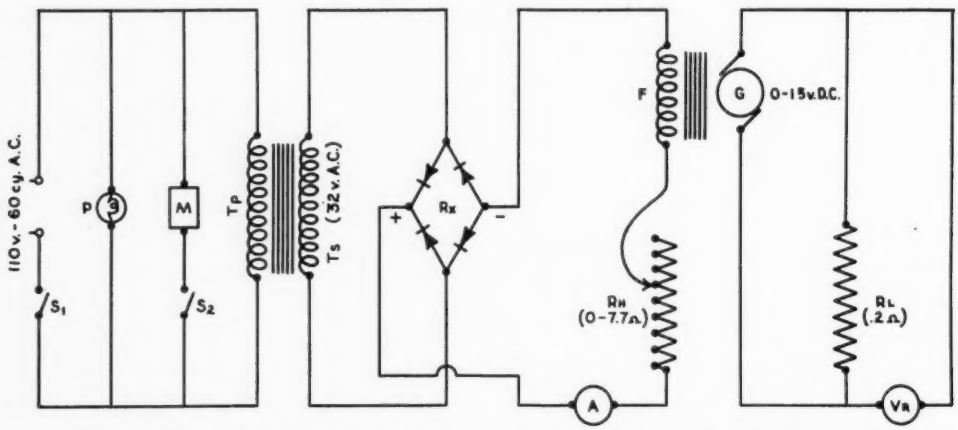

Fig. 2.—Schematic wiring diagram for the bicycle ergometer. P, Pilot light; M, motor on chart drive of voltmeter; S_1 and S_2, switches; T_p, transformer primary; T_s, transformer secondary; R_x, rectifier; F, generator field; G, generator armature; R_h, field rheostat; R_1, load resistor; A, ammeter; V_r, recording voltmeter.

Slight adjustments of this rheostat are needed constantly to meet the fluctuations of the voltage in the alternating current power lines. Larger adjustments can be made to vary the load. Since the braking action of the generator varies directly with the flow of current through the field coils, this arrangement, which is common to all ergometers based upon the electrodynamic braking action of a generator, makes it possible to adjust the load to suit the muscular strength of the subject or to meet experimental requirements and to assure accurate duplication of working conditions at all times.

The terminal brushes of the generator armature are connected to the leads of a fixed resistor (R_1, Figs. 1 and 2) which has a resistance of .2 ohms. This resistor consists of 140 cm. of 12-gauge constantan wire which has a resistivity of 49 micro-ohm-centimeter. This alloy has a temperature coefficient of resistivity of .00001 and therefore provides a constant resistance over the temperature range under which it operates. A recording voltmeter (V_r, Figs. 1 and 2) is connected to the leads of the fixed resistor to measure the drop in potential between the brush terminals of the armature. This voltage varies with the rate of pedaling and is therefore a reliable measure of the power output of the genera-

tor. This particular instrument is a 12-volt Westinghouse type GX-40 switchboard recording direct current voltmeter with a chart drive operated by a 115-volt, 60-cycle synchronous motor-driven clock. The chart drive is adjustable for speeds between three-fourths of an inch per hour and three inches per minute. Change gears are conveniently mounted in the chart mechanism and may be changed at will. The motor on the chart drive (*M*, Fig. 2) and a pilot light (*P*, Figs. 1 and 2) are operated directly from the 110-volt alternating current.

Two switches control the operation of the complete apparatus. S_1 (Figs. 1 and 2) is in the 110-volt alternating current power line and controls the entire setup. S_2 (Figs. 1 and 2) is in the chart drive circuit, making it possible to operate the machine without using the recording feature of the voltmeter.

Care of the Apparatus.—The efficiency and reliability of the ergometer depend upon the care exercised in maintaining the parts in good working order. All movable parts must be kept clean and well lubricated. The sliding contact on the rheostat and the armature brushes on the generator must be kept free of dirt and corrosion to prevent excessive electrical resistance at these points. The recording voltmeter must be installed where it will not be subjected to excessive vibration, dust, moisture, or variations in temperature. These conditions are easily met, but care must be taken to avoid the accumulation of dust inside the mechanism.

Under experimental conditions where information as to name of subject, date, time of day, etc., is to be written on the record, it becomes necessary to remove this outer case quite frequently. To avoid this inconvenience and to forestall the possibility of excessive accumulation of dust on the moving parts of the voltmeter, an opening was cut in the glass window which forms the front surface of the case and this opening was filled with a glass door which is easily opened. A small glass window was also placed in the top of the case to permit inspection of the capillary pen. These changes plus proper care have been profitable in the saving of time and in increased efficiency of operation.

Checking the Apparatus.—The bicycle ergometer should be checked frequently to guarantee efficiency of operation. A convenient method for determining the accuracy of the recorded voltage is to ride the bicycle at a uniform rate, preferably 60 cycles of the pedals per minute. This rate may be established with the aid of a metronome set to produce 120 clicks per minute. It is recommended that for pedaling at uniform rates 2 clicks per cycle be employed so alternate clicks will coincide with alternate leg movements. This recommendation is made since it was found that when a single click per cycle was used, the subject showed more fatigue in the leg which applied force in unison with the clicks.

When this procedure is followed accurately, the work rate for 60 cycles per minute is 1,500 kg. meters per minute and the generated voltage is 4.4 volts. A work rate of 40 cycles per minute is equivalent to 765 kg. meters per minute and the generated voltage is 3.1 volts. Any uniform rate is satisfactory for checking purposes, but these rates are recommended because they are the easiest to reproduce and they constitute moderate working conditions.

CALIBRATION TECHNIQUE

Calibrating the bicycle ergometer is simply a problem of determining and separating the power losses at the speeds at which the ergometer is to be used. The mechanical power input to the ergometer can be resolved into three general quantities: (1) the friction losses, (2) the electrical losses due to the resistance of the generator windings, and (3) the electrical power delivered to the external load (resistor R_1, Fig. 2) of the generator. The power for the field circuit of the generator is supplied from a separate source and therefore need not be considered in this problem. Measurement of electrical power is a simple matter but usually direct determination of mechanical power is rather difficult. The mechanical losses are most conveniently calculated by running the generator as a motor.

Measurement of Frictional Losses.—The power input to the generator when it is run as a motor consists of the electrical losses due to the resistance of the armature windings and the mechanical power delivered to the shaft to overcome the friction of the bicycle. By running the machine, with the generator as a motor, at the various speeds at which it is to be operated, the total power input at these speeds can be measured with an ammeter and a voltmeter by the following formula:

$$\text{Power input} = \text{Amperes} \times \text{volts}$$

The first step in determining the electrical losses in the motor is to block the motor so the armature cannot move and to obtain the armature resistance with an ammeter and a voltmeter by the following formula:

$$\text{Resistance} = \frac{\text{Volts}}{\text{Amperes}}$$

In larger machines the resistance of the armature circuit is not constant but appears to drop off as the current increases. However, measurements over a wide range of currents show that this particular machine does not follow this rule but has practically constant resistance. The error involved by neglecting this phenomenon and using an average value of resistance is very slight. The next step is to compute the electrical losses in the motor for all the speeds at which it is tested by the following formula:

$$\text{Power loss} = (\text{amperes})^2 \times \text{resistance of the armature}$$

These losses are subtracted from the total electrical power input at each speed to obtain the frictional or mechanical losses.

Measurement of Electrical Losses Due to Resistance of the Armature Windings.—The internal electrical losses of the generator functioning as a motor were computed in the previous step. However, the current produced by the generator, when used normally, greatly exceeds the current required to operate it as a motor under the conditions described. For this reason it is necessary to recompute these values to determine the internal electrical losses under operating conditions. The formula for obtaining these losses is the same as that used in the previous step.

Measurement of the Electrical Power Delivered to the External Load.—The power delivered to the external load is equivalent to the electrical power dissipated in the load resistor (R_1, Fig. 2). It may be computed by the formula given or from the following formula:

$$\text{Power} = \frac{(\text{Volts})^2}{\text{Load resistance}}$$

If the load resistance is not known, it may be computed by following the procedure employed in the case of the armature resistance.

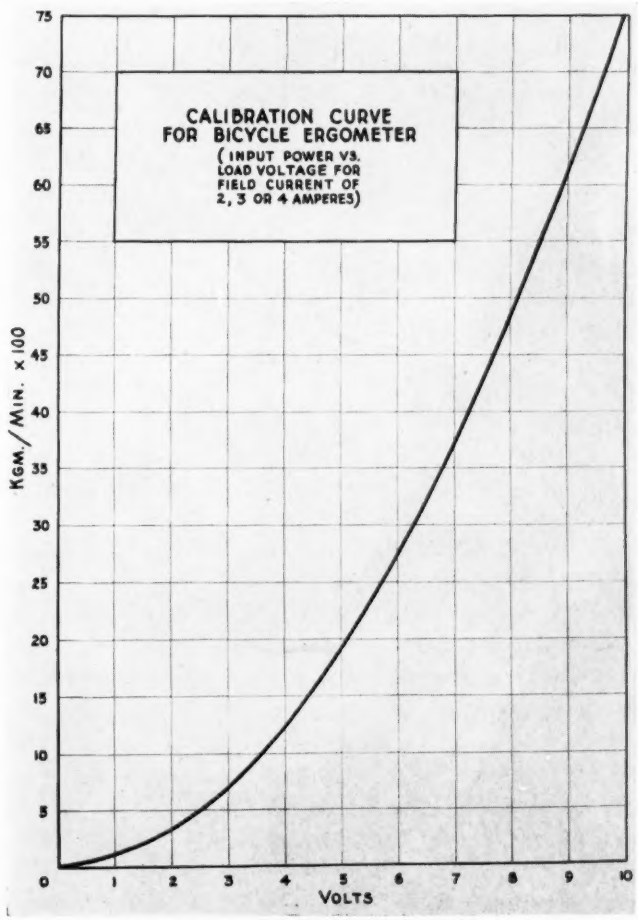

Fig. 3.—Curve for the conversion of recorded terminal voltage into work rate in kilogram meters per minute.

Drawing the Calibration Curve.—Since the power input to the ergometer is equal to the sum of the mechanical losses, armature losses, and load resistor losses, these values are summed up and this quantity is the total power input to the generator system for the specified terminal voltage. By computing a number of these quantities and plotting them against their respective voltages, a calibration curve may be drawn (see Fig. 3).

Power values computed in this manner will, of course, be in watts and if the curve is to be in terms of kilogram meters per minute, the power values must be multiplied by the conversion factor of 6.15. The figures presented in Table I are based upon the calibration curve shown in Fig. 3. They are used to convert terminal voltage to work rate or power in kilogram meters per minute for field currents between 2 and 4 amperes.

TABLE I

WORK RATE EQUIVALENTS IN KILOGRAM METERS PER MINUTE FOR GENERATED TERMINAL VOLTAGE (2 TO 4 AMPERES FIELD CURRENT)

VOLTS	KG.M./MIN.	VOLTS.	KG.M./MIN.
2.0	340	6.0	2,760
2.2	400	6.2	2,940
2.4	470	6.4	3,130
2.6	550	6.6	3,330
2.8	630	6.8	3,530
3.0	720	7.0	3.740
3.2	810	7.2	3,950
3.4	910	7.4	4,170
3.6	1,020	7.6	4,400
3.8	1,130	7.8	4,630
4.0	1,250	8.0	4,870
4.2	1,370	8.2	5,120
4.4	1,500	8.4	5,370
4.6	1,640	8.6	5,630
4.8	1,780	8.8	5,890
5.0	1,930	9.0	6,160
5.2	2,080	9.2	6,440
5.4	2,240	9.4	6,720
5.6	2,410	9.6	7,000
5.8	2,580	9.8	7,290

TECHNIQUE FOR MEASURING WORK RATE (POWER) AND WORK OUTPUT

The bicycle ergometer described in this paper is adaptable to many uses in research relating to the working efficiency of the human body. Most investigations in this field of experimentation have been concerned with the physiologic effects of working at a constant rate for prolonged periods. The automatic recording feature of the apparatus makes it possible to determine constancy of work rate and total amount of work performed. Other studies have attempted to approximate the optimum rate and load for working under various conditions. The ergometer provides an accurate method of measuring the work rate, work load, and total work output.

Recently, studies in the field of physical fitness have employed the bicycle ergometer to measure work rate and total output during short periods of working at maximum speeds. Since dynamic physical fitness may be defined as the capacity to do work, the ergometer provides reliable and valid criteria of this type of physical fitness. The findings of these studies are as yet inconclusive, but they seem to indicate that in some cases maximum total work output for a period of about two minutes is in itself a valid criterion of dynamic physical fitness. Other findings indicate that work output relative to body size and strength is one of the most valid measures of dynamic physical fitness.

The chief purpose of the bicycle ergometer in all these investigations has been to obtain a reliable estimate of the work rate and work output. The technique for obtaining these measures from continuous voltage recordings as obtained from the bicycle ergometer is described in the next paragraphs.

Determining Work Rate (Powe).—A typical voltage curve obtained by pedaling the bicycle ergometer at top speed for two minutes is reproduced in Fig. 4. The tracing is a continuous recording of the generated voltage. The small fluctuations coincide with vibrations in the apparatus and the uneven application of force to the pedals and may be disregarded. The larger fluctuations represent changes in work rate due to fatigue and alterations in effort on the part of the subject.

From the record shown in Fig. 4 it is possible to obtain the generated voltage at any instant during the two-minute working period represented by this tracing. By referring to Table I, this instantaneous voltage can be converted to work rate or power in kilogram meters per minute. This measure, in itself, is interesting and of value in certain types of investigations, but its chief value lies in its use for determining work output for definite working periods.

Determining Work Output.—The record shown in Fig. 4 also represents the fluctuations in work rate for a working period of two minutes. To obtain the work output for all or any part of the two-minute period, it is necessary to redraw this curve so that the area under all parts of the curve will represent the work done (see Fig. 5). This is essential because the generated voltage is not a linear function of the power input to the machine.

To accomplish this, the following steps must be carried out: First, obtain voltage readings at regularly spaced moments throughout the working period and by referring to Table I convert these voltage readings into work rates in kilogram meters per minute. Next, replot the curve with work rate on the y-axis and time on the x-axis. The result is a work curve, and the area under the curve, being the product of work rate and time, is proportional to the work output. Therefore, the final step is to measure the area under all or any part of the curve with the aid of a planimeter and to multiply this area by the appropriate conversion factor. The resultant will be the work output for the period of time represented by the section of the work curve marked off.

Since the conversion factor mentioned in the previous paragraph is dependent upon the scales used in plotting the work curve, the following illustration is presented to clarify the last-mentioned step: Let us assume that our replot is made upon cross-section paper which is ruled off in millimeters and that 1 cm. on the vertical axis represents 100 kg. meters per minute work rate. Now if the horizontal axis is laid off so that 10 cm. represents one minute of elapsed time, 10 sq. cm. of area under the curve will be equal to 100 kg. meters of work and 1 sq. cm. will be equal to 10 kg. meters of work. The value 10 then becomes the conversion factor when the planimeter is adjusted to measure area in square centimeters.

This method for measuring work output is recommended when a planimeter is available. However, this instrument is not always accessible so that the following substitute method may prove more feasible: Proceed by marking off five-second intervals on the voltage tracing. Then obtain an average voltage

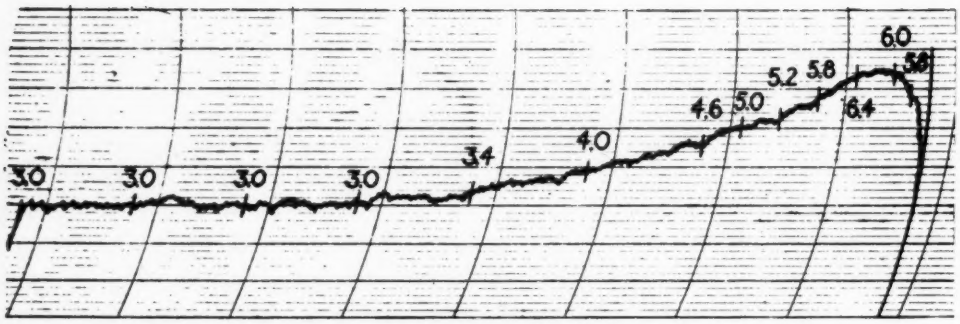

Fig. 4.—A typical voltmeter recording showing the continuous generated voltage during a two-minute working period on the bicycle ergometer.

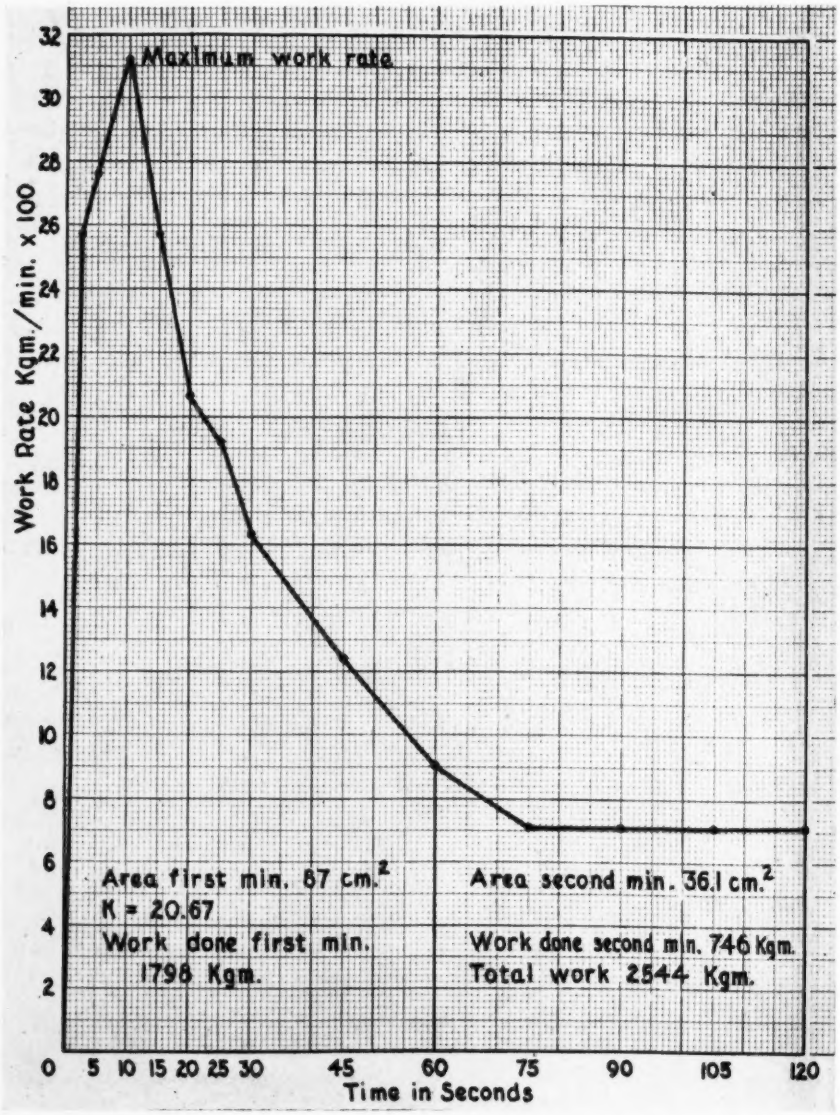
Maximum work rate
Work Rate Kgm./min. x 100
Time in Seconds
Area first min. 67 cm.²
K = 20.67
Work done first min.
1796 Kgm.
Area second min. 36.1 cm.²
Work done second min. 746 Kgm.
Total work 2544 Kgm.

Fig. 5.—The plotted work curve for the voltmeter recording shown in Fig. 4.

reading for each interval, and by referring to Table I convert these readings to work rate values. The mean of these values will then represent the average work rate during the working period. The product of this average work rate and the elapsed time in minutes is a reliable estimate of the work performed. While this approach is not as accurate as the first-mentioned method, especially when the voltage curve fluctuates markedly, the results do not, as a rule, deviate by more than 2 per cent from planimeter measurements. This is well within the expected error for similar measurement techniques.

Using the Ergometer for the Measurement of Physical Efficiency.—The bicycle ergometer lends itself well as a method of work in determining physical efficiency by the oxygen consumption method. The most satisfactory work rate has been found to be 1,500 kg. meters per minute for one minute. This means a pedal rate of 60 revolutions per minute controlled by having the subject work in rhythm with a metronome set to produce 120 clicks per minute. The limiting factor in the determination of physical efficiency by this method is the spirometer from which the oxygen is breathed. To provide enough oxygen for a greater work rate than 1,500 kg.-meters per minute for one minute necessitates enlarging the outlet of the spirometer. This in turn results in a large dead air space and causes rebreathing of expired air. The respiration of the subject is so affected by this rebreathing that the experiment cannot be carried to completion successfully.

The physical efficiency of the average individual, as measured by the oxygen consumption method using the bicycle ergometer as the method of work, has been found to vary between 20 to 25 per cent.

SUMMARY

The construction, calibration, and use of an alternating current electrodynamic brake bicycle ergometer are described. In addition, techniques for measuring work output and maximum work rate are discussed. Instructions for the care and standardization of the machine are included.

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A DEVICE FOR RECORDING THE RESPIRATION OF ANESTHETIZED LABORATORY ANIMALS

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THE usual pneumograph for recording the respiration of laboratory animals requires a sensitive and airtight tambour and has the disadvantage that it may be dislodged from a fixed position on the chest. This device may also fail to record diaphragmatic breathing if, due to increased anesthesia, the intercostal muscles become paralyzed. The pleural cannula* has the disadvantage of producing some trauma to the pleura or lung, and if the tambour is not airtight, unsuspected pneumothorax may result. Other respiratory recording devices tend to be complicated and cumbersome.

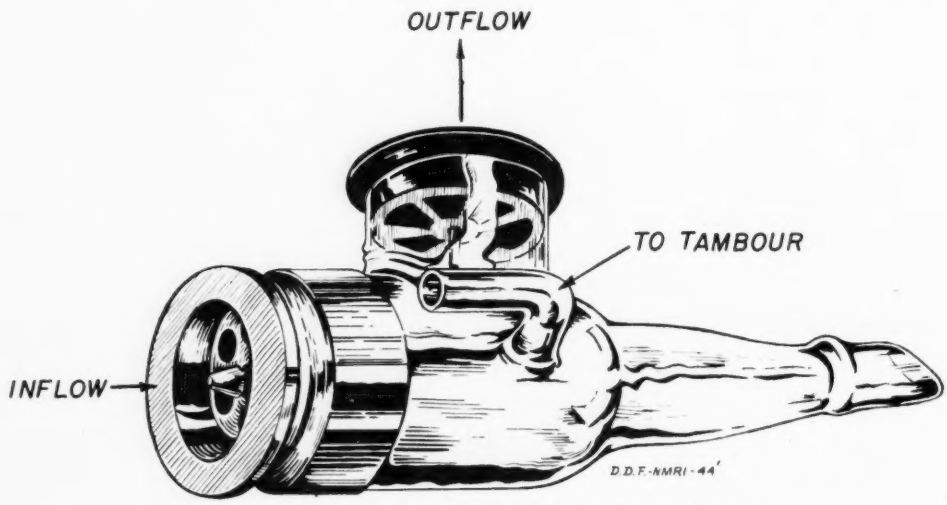

Fig. 1.—Modified tracheal cannula (two-thirds actual size).

Several years ago a suitable tracheal cannula modified for recording respiration was constructed using a two-way system of wedge-shaped flutter valves with a side arm connecting to a tambour. When used in large dogs, however, the valves did not allow for adequate respiratory exchange. With the advent of improved flutter valves, such as are now used in aviators' oxygen masks (M-1 and A-10 valves),† an accurate and efficient device can be easily constructed.

*Mendenhall, W. H.: Science **86**: 129, 1937.

†Acushnet Processing Co., Bridgeport, Conn.

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Due to the present shortage of metallic materials, the model pictured in Fig. 1 is made almost entirely of glass. A more durable recorder could be made of brass or plastic. The recorder produces a constant record of respiration with

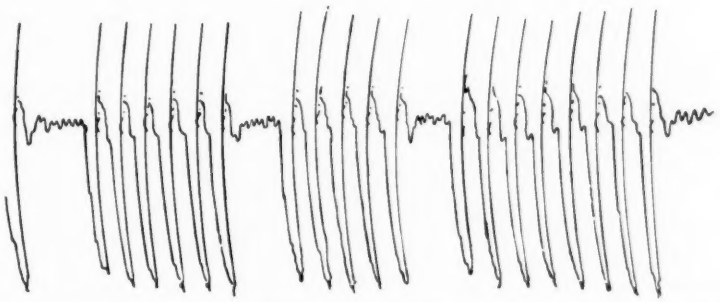

Fig. 2.—Typical recording of respiration and pulmonary pulse (actual size).

any of the usual laboratory tambours (Fig. 2). Inspiration is recorded by the downstroke and expiration by the upstroke. Since both valves are closed between respiratory cycles, the pulmonary pulse usually is recorded. This may at times be advantageous. By means of a slip joint on the inflow flutter valve, the composition of the atmosphere supplied to the animal may be varied at will.

PHYSIOLOGICAL SALINE WITHOUT WEIGHINGS

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THIS time-saving and labor-saving method of preparation, well adapted to field use, was developed to expedite filling of the hospital's large requirements for saline for lavages, rinsing of special surgical equipment, etc. Subsequently the saline so prepared was found entirely suitable for all laboratory and clinical uses as well, and the method has been employed with satisfaction for the past twelve years.

The chemical principle made use of, peculiar to sodium chloride, is its slight solubility change with change of temperature. The solubility plotted against temperature yields a straight line,[1] and over the extreme temperature range from 0 to 100° C. rises only from 35.7 to 39.8 Gm. per 100 Gm. water.[2] For a 10° temperature interval (ordinary room temperature range) the over-all change in solubility is approximately 1 per cent of the total, and the ultimate dilution of the finished saline is such that the actual weight of salt varies only by 0.01 Gm. per 100 c.c. The several concentrations which have been recommended by various authorities as "normal saline" (from 0.85 to 0.90 per cent) are much farther apart than this, differing in fact by more than 5 per cent, or 0.05 Gm. per 100 c.c.

A saturated solution of sodium chloride in distilled water, above a thick layer of excess salt, is maintained in a large stock bottle with salt or water added as needed. The saturated brine is filtered off from time to time into another stock bottle and the saline is prepared from this filtrate by dilution.

Since 100 Gm. of water containing over 35 Gm. of sodium chloride no longer occupied 100 c.c., it was necessary to work out the new weight-volume relationships and determine by accurate gravimetric methods the salt content per unit of volume. The calculations were made for a physiological saline of 0.90 per cent concentration.

Dilution of 2.60 c.c. of filtered saturated brine to 100 c.c. with distilled water produces a saline containing exactly 0.90 Gm. sodium chloride per 100 c.c. In titration with silver nitrate, using chromate as indicater, 2.00 c.c. of this saline are equivalent to 10.91 c.c. of M/35.46 silver nitrate.

Over the temperature range from 0 to 47° C., or from 32 to 117° F., the total change in concentration of the finished saline still remains no greater than that between 0.85 and 0.90 per cent saline. For nearly absolute constancy of concentration, the volume of brine used can be increased by 1 per cent for each 10° interval below 18° C. and decreased by 1 per cent for each 10° interval above 28° C. For all practical purposes, such correction is unnecessary.

Sterilization of saturated brine by autoclaving or boiling does not affect the concentration, as loss of water leaves the solution still saturated. On the other hand, the application of heat in preparing the brine from the dry salt is

From the Wm. H. Maybury Sanatorium (Detroit Municipal Tuberculosis Sanatorium). Received for publication, Dec. 8, 1944.

of no particular benefit due to the special solubility-temperature relationship described. Agitation, to get the heavy layers off the bottom, is the main necessity in obtaining saturation; a shaking machine may be used, but an occasional roll or shake of the stock bottle by hand is effective.

A further advantage in the use of saturated soduim chloride brine is the ease of making a saline from some other solution (nutrient or antiseptic) with only negligible dilution of the other component; sterile filtered brine, in the small volume required, again 2.60 c.c. per 100 c.c., can be added quickly with sterile technique.

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MEDICAL ILLUSTRATION

LIGHT SOURCE FOR FLUORESCENT MICROSCOPY

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A STRONG source of violet light is desirable in using the fluorescent method of detecting tubercle bacilli. Most recent work seems to have been done with the 100-watt AH4 mercury lamp. However, Kloeck and Sweany[1] have obtained much better results with the 250-watt CH5 lamp. Both of these lamps are made with a quartz light capsule sealed in a clear tubular bulb. The CH5 lamp is considerably larger than the AH4.

Another lamp which has several useful features is the 100-watt CH4 lamp.* In this lamp the quartz capsule is sealed in a bulb with an aluminum reflector, similar to a sealed beam headlight. The construction is such that a strong spot of light is obtained. In this arrangement the lamp supplies its own housing and efficient optical system. It is necessary only to support the lamp from a laboratory stand and to supply current from the proper transformer.

Some comparative tests have been made which should be helpful in using these lamps. A metal plate with a 0.5 mm. hole drilled through it was fitted into the mechanical stage of the microscope. A selenium photocell was fastened above this hole and connected to a microammeter. With this apparatus, the light intensity over a small area of the field could be determined. All readings were made with a Corning No. 5113 violet filter fastened under the condenser.

The distance between the CH4 lamp and the microscope mirror was varied and the photocell readings noted. Over a range of from 10 to 25 cm. the readings were almost constant.

When using the CH4 lamp it will be noted that the pattern of the field as observed in the top of the condenser consists of a black center surrounded by a broad ring of light. The black center corresponds to the opaque end of the light capsule mounted in the bulb. The ring of light is not uniform in intensity, but an intense area can be brought into the field by adjustment of the microscope mirror.

Similar tests were made with the AH4 lamp. By allowing this lamp to burn freely in the air it was found the intensity dropped rapidly as the lamp was moved away from the microscope. At 3 cm. from the microscope mirror the intensity was nearly equal to the CH4 lamp, whereas at 10 cm. the intensity was 25 per cent of this value.

Dr. H. C. Sweany, of the Chicago Municipal Tuberculosis Sanitarium, kindly made their CH5 lamp available for measurement. This lamp was

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*Listed by General Electric Co. and Westinghouse Electric and Manufacturing Co.

mounted in a housing with a mirror in the rear and a condensing lens in front. The photocell gave readings nearly equal to that of the CH4 lamp.

The use of an aluminized microscope mirror has been suggested to increase the illumination. A nonfluorescent liquid, such as water, placed between the condenser and slide also increases the illumination. However, the photocell indicated that neither of these methods would cause a 10 per cent increase. Visual tests indicated that the increased illumination was not especially noticeable.

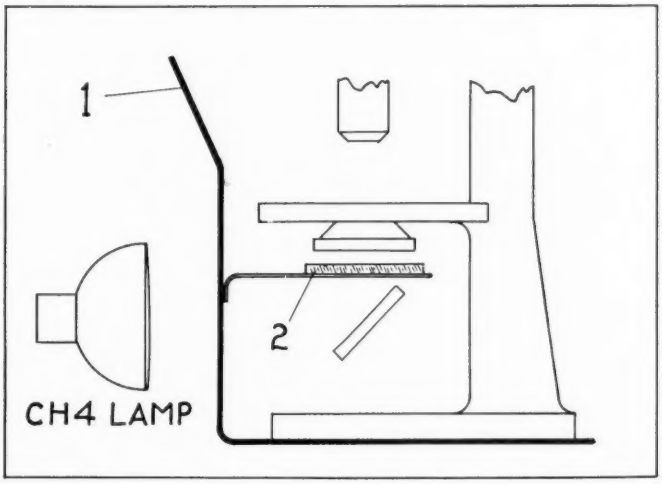

Fig. 1.—Simple apparatus for fluorescent microscopy.

The following notes are the result of experience obtained in the University of Chicago Clinics from tests of the fluorescent method for detecting tubercle bacilli.

An ordinary microscope can be readily converted into a fluorescent microscope, as shown in Fig. 1. The CH4 lamp is sufficiently intense for binocular vision. A simple light shield, *1*, is required. Filter 2, made of Corning No. 5113 violet glass, is mounted under the condenser. A filter of Corning No. 3486 yellow is supported under the eyepiece. Both filters are of "standard" thickness.

The stained slides are readily scanned with the low-power objective, and final identification of the tubercle bacillus is made with the oil immersion lens.

To avoid the use of oil, a 45 × N. A. .95 objective was tested. The resolving power of this lens was inferior to the oil lens but might be considered satisfactory. It is important that such a lens not be corrected for cover slip thickness.[2]

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A NOTE ON INFRARED PHOTOGRAPHY OF CUTANEOUS ARTERIAL "SPIDERS" AND HEREDITARY HEMORRHAGIC TELANGIECTASES

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THE infrared technique has been used widely to give photographic accentuation to dark red or bluish structures on or near the surface of human skin. Collateral venous channels not visible to the unaided eye have been demonstrated over the abdomen in cirrhotic patients with ascites; and the large networks of veins over the breasts and anterior thoracic wall of pregnant women have been depicted in infrared photographs, whereas they do not appear in ordinary black-and-white pictures or appear but faintly.

During a study of several hundred examples of the cutaneous arterial "spiders"[1, 2] it occurred to me that the opposite effect might be obtained, namely, infrared photographs might eliminate bright red structures which were recorded in a satisfactory manner by means of ordinary photography. Such proved to be the case. The arterial "spider" of the skin has a bright red color which depends on the abundant supply of arterial blood brought unusually near the surface of the skin in thin-walled vessels. In Fig. 1 black-and-white and infrared photographs of the same area of the forearm and antecubital region of a patient with advanced cirrhosis are compared. A number of "spiders," some large and some small, stand out in clear detail in the black-and-white photograph. They do not appear, except for a faint ghost marking the site of the body of the spider, in the infrared photograph. This photographic comparison has been carried out several times with precisely the same results.

Although the arterial nature of the vascular "spider" of the skin has been known for more than fifty years, and pulsation can be detected in the larger ones, this technique offers a diagnostic aid in establishing the nature of small, nonpulsatile, or atypical lesions.

In Fig. 2, the lesions of hereditary hemorrhagic telangiectasia (Osler's disease) are compared by the same technique. It is readily apparent that the same phenomenon occurs when this type of lesion is photographed by the infrared method as when the arterial "spider" is photographed in black and white, namely, it does not appear in the picture. This is of interest since the color of the smaller punctate lesion of Osler's disease is usually more of a red-blue or violaceous color than the arterial "spider," which is regularly a bright scarlet color, from the oxyhemoglobin. In both cases this method gives additional evidence that the vessels chiefly affected are on the arterial side of the capillaries and carry arterial blood. It is possible to bring out the gradations in color and the contrasts between the "spider" and the hereditary telangiectases by the use of Kodachrome and other color films, but the exact reproduction of

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Fig. 1.—A comparison of black-and-white (left) and infrared (right) photographs of the forearm of a patient with cirrhosis showing the failure of the lesion to appear in the infrared photograph.

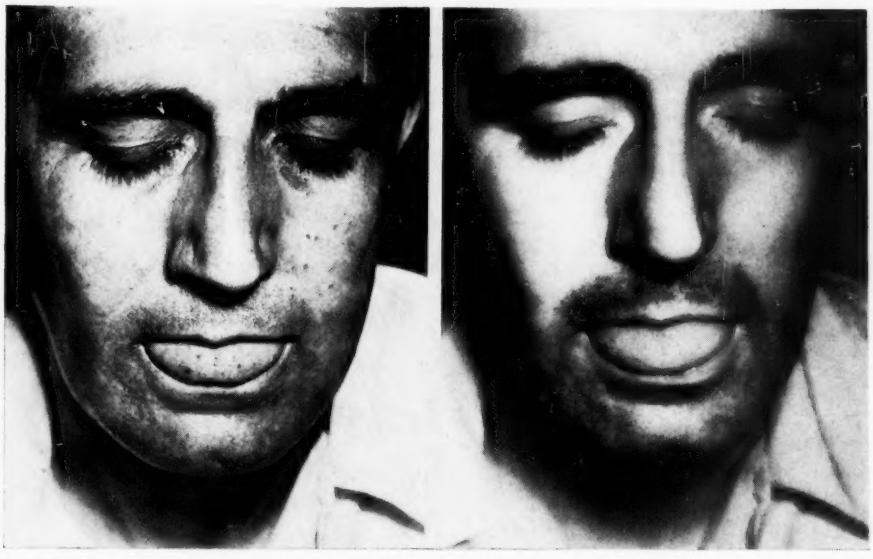

Fig. 2.—A comparison of black-and-white (left) and infrared (right) photographs of a person with Osler's disease—*hereditary hemorrhagic telangiectasia.* Lesions on the tongue and cheek fail to appear except as ghost marks in the infrared photographs.

colors is still difficult technically. For that reason, comparison of black-and-white and infrared photographs offers a simple method of establishing the arterial or venous nature of vascular lesions of the skin and mucous membranes.

CONCLUSION

The fact that the arterial "spider" of the skin does not appear in infrared photographs suggests a use in segregating it from venous telangiectases, though they will not serve to differentiate it from the telangiectasis of Osler's disease.

I am indebted to Mr. Joseph B. Homan, Assistant Professor of Medical Photography, for taking the pictures.

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BOOK REVIEWS

A Textbook of Pathology. By *Robert Allan Moore,* Edward Mallinckrodt Professor of Pathology, Washington University School of Medicine. W. B. Saunders, Philadelphia, Pa. Price $10.00. Cloth with 1,338 pages with 513 illustrations, 34 in color.

This textbook of pathology, the latest to appear in the American literature, is well printed and illustrated and should take its place as one of our foremost texts. The author has made it more attractive than some textbooks by including a considerable number of colored plates, as well as radiographs, photographs of patients, and reproductions of frontispieces from classical monographs, thus reflecting his wide experience both in the teaching and study of pathology in this country and abroad. As a result the book lacks some of the provincialities too frequently manifested in certain textbooks. The arrangement of the book includes both general and special pathology, and the emphasis throughout is predominantly on etiology. In association with the discussion of the various pathologic entities there is usually a clinicopathologic correlation. The author has also assembled at the end of each chapter a selected list of references, most of which are relatively recent. Students using these, therefore, can do so with the assurance that they are studying pathology as a developing science. Throughout the book the author demonstrates his purpose of keeping abreast of the current literature and of describing pathologic changes not only in their relationships to morphology, but also to biochemistry, physiology, bacteriology, and clinical medicine. As with every textbook, the discussions have been necessarily restricted, but the author has shown good discrimination in his selection of material. The book may be recommended as a reliable and well-planned textbook with the particular merit of emphasizing dynamic aspects of pathology.

P. R. C.

Familial Susceptibility to Tuberculosis: Its Importance as a Public Health Problem. By *Ruth Rice Puffer,* Dr.P.H., Tennessee Department of Public Health. Harvard University Press, Cambridge, Mass., 1944. Price $2.00. Cloth with 106 pages.

Isolation of the diseased patient—the prevention of contact—has been the principal method used in public health programs for the control of tuberculosis. But, as Dr. Puffer states in her introduction: "Although isolation reduces the opportunities of exposure of the population, this measure has not been sufficient to prevent the majority of the population in many sections of the country from becoming infected before reaching adult life. Since we cannot prevent all contact with tubercle bacilli, we need to go deeper into the problem to understand the conditions under which infection results in disease." She attempts

to do this in part by analyzing the importance of familial susceptibility to tuberculosis and suggesting how this information may be used in an attempt to make the tuberculosis control program more effective.

The literature is reviewed and new data from the Williamson County (Tennessee) Tuberculosis Study are presented. Her analysis of data for siblings, consorts, parents, and children of tuberculous patients suggests that hereditary or familial susceptibility is a determinant in the development of the disease. The families of consorts were studied as one of the control groups. Dr. Puffer emphasizes that the development of tuberculosis must be studied as a manifestation of a patient's total life experience. If the attack rate is studied in children of tuberculous patients, for instance, it is not enough to terminate the study when the child leaves his parents' home to establish one of his own. She points out that "while children of a sputum-positive parent developed tuberculosis very frequently in childhood, children of a parent with tuberculosis other than sputum-positive developed the disease frequently in adult life. By 50 years of age approximately the same proportion of both groups of children had developed tuberculosis."

The data available for this study indicate that the incidence of tuberculosis is as great among susceptible families as it was before the turn of the century. Dr. Puffer suggests that since the tuberculosis death rates of female children of tuberculous parents are particularly high, these families tend to die out. The decline in tuberculous death rates, therefore, may be due in large part to the reduction of susceptible families in the population.

In most existing public health programs, only the household associates of a patient who develops tuberculosis are examined and followed. The studies summarized in this monograph indicate that these programs should be augmented so that "siblings, parents, and children of persons known to have tuberculosis or to have had the disease in the past be examined and followed irrespective of their present household."

Dr. Puffer presents her argument in a simple and convincing manner. Her book should prove as stimulating and helpful to the clinician as to the public health physician. It is a worthy companion to the other Harvard University monographs in medicine and public health.

C. V. M.

The Avitaminoses: The Chemical, Clinical and Pathological Aspects of the Vitamin Deficiency Diseases. By *Walter H. Eddy*, Ph.D., Emeritus Professor of Physiological Chemistry, Teachers College, Columbia University; and *Gilbert Dalldorf*, M.D., Pathologist of the Grasslands and Northern Westchester Hospitals, Westchester County, N. Y. Third Edition. The Williams & Wilkins Company, Baltimore, 1944. Price $4.50. Cloth with 438 pages.

Medical Uses of Soap. Edited by *Morris Fishbein*, M.D., J. B. Lippincott, Philadelphia. Price $3.00. Cloth with 182 pages, 41 illustrations.